safety and efficacy of assisted reproductive techniques in male infertility

Aukje Meijerink

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For reasons of consistency within this thesis, some terms have been standardised throughout the text. As a consequence the text may differ from the articles that have been published.

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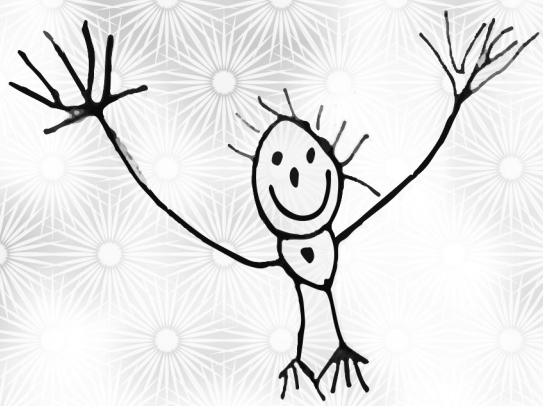
Contents

1

Introduction and aims of this thesis



One out ten couples with the wish to conceive will face infertility. Infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild, et al., 2009). These couples are then referred by their general practitioner to the fertility clinic to seek help.
In approximately 40% of infertile couples a male factor is involved (Hull, et al., 1985). Male infertility presents mostly with decreased sperm quality.

Spermatogenesis & Azoospermia

Spermatogenesis takes places in the testes, which contain two anatomical units: first a network of seminiferous tubules containing Sertoli cells and second the interstitium containing Leydig cells as presented in Figure 1 (Page, et al., 2008).

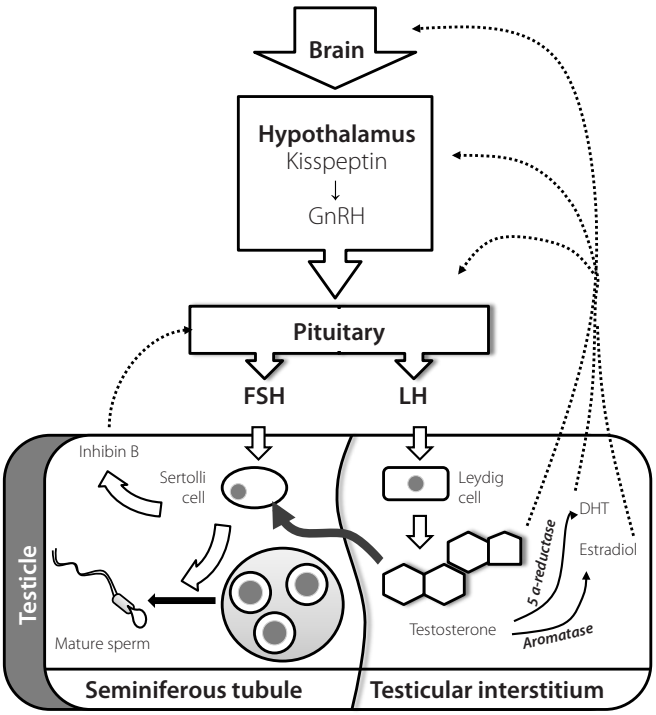


Figure 1 Endocrinology of spermatogenesis

Spermatogenesis is regulated by a hormonal axis comprising three anatomical components: hypothalamus (located in the brain), pituitary gland (located in the brain) and the testicles. The hypothalamic-pituitary-testicular axis, regulates an endocrine loop with negative feedback of downstream hormonal products. Testicular production of testosterone and inhibin B is carefully regulated by gonadotropins produced by the pituitary gland. Gonadotropin production is under the direct control of pulsatile gonadotropin releasing hormone (GnRH) secretion from the hypothalamus. The main hormones that actually control spermatogenesis are follicle stimulating hormone (FSH) and testosterone. FSH receptors are present in Sertoli cells and spermatogonia, and androgen receptors are present in Sertoli cells, Leydig cells, and peritubular myoid cells. After spermatogenesis takes place in the testis (Page, et al., 2008), mature sperm cells are stored in the epididymis (Figure 2). During ejaculation, sperm cells from the epididymis accompanied with prostate fluid and secrete of the seminal vesicle form the ejaculate (Nap, 2013). Sometimes there are no spermatozoa present in the ejaculate at all, a condition called azoospermia. Azoospermia is diagnosed as the cause of the infertility in 5-10% of infertile couples, and also found in one percent of the general population (De Croo, et al., 2000, Jarow, et al., 1989).

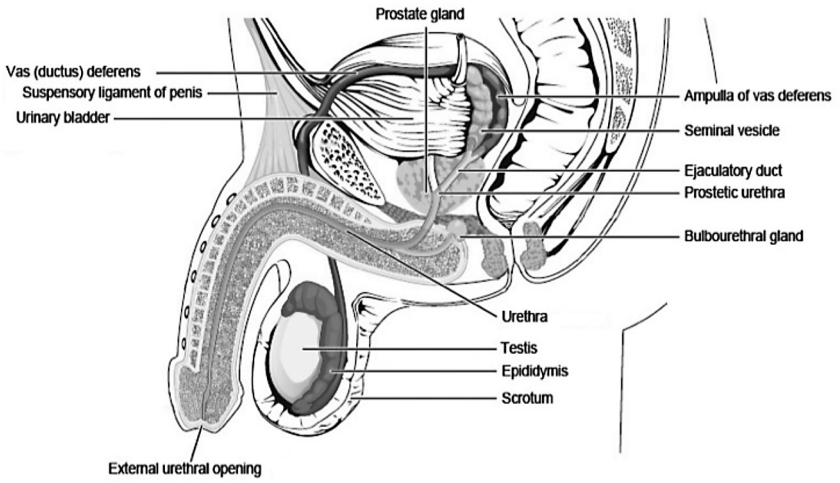


Figure 2 Anatomy of the male reproductive tract

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The most used classification of azoospermia is based on its aetiology: a transport problem of sperm cells after they leave the testes (obstruction) or a disturbed spermatogenesis (no obstruction). Obstructive azoospermia (OA) is diagnosed in 30-40% of the cases while non-obstructive azoospermia (NOA) is found in 60-70% of the azoospermic males (Ezeh, 2000, Matsumiya, et al., 1994).

Obstructive azoospermia

In OA, normal spermatogenesis is assumed, but due to an obstruction of the spermatic chords (vas deferens) in the more distal parts of the epididymis, sperm cells are not found in the ejaculate. Causes of OA are among others: epididymitis (inflammation of the epididymis); testicular trauma resulting in damage; status after vasectomy and absence of spermatic cords (congenital bilateral absence of vas deferens). This last condition is found in males suffering from cystic fibrosis or in males who are carrier of this gene defect. Histological normal or mild hypospermatogenesis is often found in these men with OA. Therefore, sperm cells can be retrieved by puncturing the epididymis with a needle, called percutaneous epididymal sperm aspiration (PESA), for use in fertility treatment, see next paragraphs.

Non-obstructive azoospermia

In NOA, the lack of sperm cells in the ejaculate is due to a qualitative and/or quantitative extremely disturbed spermatogenesis. Generally, these men present with low testicular volume and high levels of FSH, see Box 1 for the definition of NOA (Adamopoulos and Koukkou, 2010, Jungwirth, et al., 2012).

Known causes of NOA are iatrogenic damage (radiation or chemotherapy); testicular torsion; orchitis (inflammation of the testes); cryptorchidism (a condition in which one or both of the testes fail to descend from the abdomen into the scrotum) or genetic abnormalities (Y-chromosomal AZF-deletions or Klinefelter syndrome). Y-chromosomal deletions are observed in about three percent of males diagnosed with NOA (Johnson, 1998, Pryor, et al., 1997, Skaletsky, et al., 2003). Unfortunately, in most men with NOA the cause of disturbed spermatogenesis remains unexplained.

Box 1 Definition non-obstructive azoospermia

Azoospermia without evidence of obstruction with either:
- Small testes (volume per testis <15 ml)

and / or
- Elevated FSH level (>10 IU/L)

and / or
- Decreased Inhibin B (<150 ng/L)

In order to obtain sperm cells for fertility treatment in NOA men, a testicular biopsy (TESE, testicular sperm extraction) can be performed. Several histological stages can be observed when taking a testicular biopsy. In case of Sertoli-Cell-Only syndrome spermatogonial stem cells are absent (germinal cell aplasia) which will lead to an unsuccessful sperm retrieval when TESE is performed (Levin, 1979). When spermatogonia are present but spermatogenesis development stops at some point during maturation, we call this maturation arrest. This happens most often at first meiotic prophase and no sperm or scarcely sperm cells, not appropriate for fertility treatment, can be found (Levin, 1979). In other cases there is focal spermatogenesis, which is a mixture of tubules with Sertoli-Cell-Only and tubules with active spermatogenesis (Levin, 1979).

Azoospermia: Treatment and health of offspring

Sperm cells obtained by PESA or TESE can be used for intracytoplasmic sperm injection (ICSI) treatment in order to conceive a genetically own child. From the moment PESA-ICSI and TESE-ICSI were introduced into daily practice in the early nineties (Devroey, et al., 1994, Nagy, et al., 1995, Tournaye, et al., 1994), the Dutch board of gynaecologists (NVOG) and embryologists (KLEM) expressed their concerns about the possible short and long term effects on the health of children conceived by surgical retrieved sperm. Hence, in 1996 a moratorium was set on the use of surgical retrieved sperm for ICSI treatment. For couples facing azoospermia it was not possible to receive fertility treatment in the Netherlands. This resulted in couples seeking fertility treatment abroad. The first international follow up report of ICSI children born from OA and NOA fathers, indicated that there were no increased health risks in terms of major birth defects compared with ICSI children conceived with ejaculated sperm (Bonduelle, et al., 1998). Therefore, after approval of the Central Committee on Research Involving Human Subjects (CCMO) in 2001, ICSI using epipidymal sperm was restarted but under strict regulations. Research into the quality of epididymal sperm and prospective follow up of children born after these procedures were performed in order to evaluate health effects. At the Radboud university medical center, a study of quality of epididymal sperm in azoospermia, reported highly elevated rates of sperm DNA damage and chromatin condensation defects in epididymal sperm (PhD thesis L. Ramos). However, sperm selection based on motility showed similar quality to ejaculated sperm (Ramos, 2004). In a follow up study of Woldringh et al. the prevalence of birth defects and development of children born after PESA-ICSI were studied. The group reported that ICSI with epididymal sperm did not lead to more still births or congenital malformations in comparison to children born after IVF. Moreover, ICSI with ejaculated sperm did not lead to poor development of children compared with a Dutch reference group of children born after IVF or ICSI with ejaculated sperm (Woldringh, 2011). In 2007, besides PESA-ICSI also TESE-ICSI was allowed in research settings in Radboud university medical center (Nijmegen) and Academic Medical Center (Amsterdam). The CCMO protocol focussed on the safety aspect of TESE and the health of the children born

from NOA men. At that time insufficient data about late developmental effects in children born after TESE-ICSI were available. Two studies described pregnancy and neonatal outcome of children born after TESE-ICSI short after birth, either by data analysis (Vernaeve, et al., 2003) as well as by physical examination (Ludwig and Katalinic, 2003). No prospective studies had been performed regarding behavioural, cognitive, motor and physical performance of children born after TESE-ICSI older than two years of age. The Central Committee on Research Involving Human Subjects (CCMO, The Hague, the Netherlands) approved a study protocol studying the behavioural, cognitive, motor and physical performance of children born after TESE-ICSI, at the age of five. After a preliminary report of this study (May 2014) regarding the prevalence of birth defects in these children, and a Quality Standard regarding ART with surgical retrieved sperm developed by the Dutch board of gynaecologists (NVOG) , urologists (NVU) and embryologists (KLEM), in June 2014 the moratorium on TESE-ICSI was lifted by Mrs. E.I. Schippers, Minister of Health, Welfare and Sport (NVOG, 2013, Staatscourant, 2014). Figure 3 presents an overview of the availability (introduction and re-introduction) of assisted reproductive techniques in the Netherlands.

The azoospermic couple: Counselling

Only in about half of TESE procedures, sperm retrieval is successful in men with NOA (Chan and Schlegel, 2000, Colpi, et al., 2005, Tournaye, 2010). In men with OA who had a failed PESA, TESE is more successful (Omurtag, et al., 2013) than in men with NOA. Preference for epididymal sperm upon testicular biopsy is based on the quality of sperm and risks of the surgical procedure. Taking a testicular biopsy carries a small risk of complications, including loss of significant amount of testicular tissue, haematoma, inflammatory changes and permanent devascularisation (Schlegel and Su, 1997). Even though the pregnancy rate after TESE-ICSI is encouragingly high, it is lower than in men with normal spermatogenesis

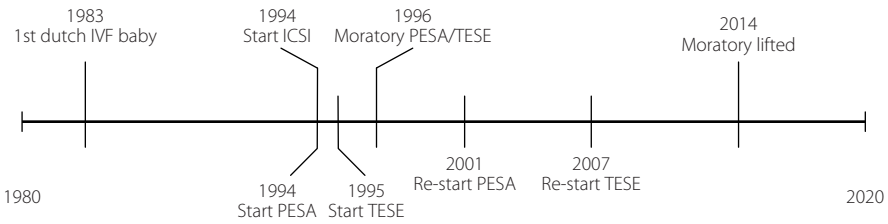

Figure 3 Timeline of availability and introduction of assisted reproductive techniques in The Netherlands

(Nicopoullos, et al., 2004, Tournaye, 2010). Figure 4 presents the steps an azoospermic couple has to undergo during the fertility treatment.
For those couples with failed testicular sperm retrieval or those who failed to conceive after TESE-ICSI, insemination using donor sperm, adoption or becoming foster parents are other options to fulfil their child wish. In order to involve couples in their treatment and help them making their own choices by shared decision making, the fertility professional should present the treatment options and expected outcome clearly (Legare, et al., 2014). Nowadays, counselling couples about their chances of successful sperm retrieval by TESE and a successful TESE-ICSI outcome, in terms of treatment resulting in a live born child, is mandatory. Couples have to consider whether it is worth for them to start the intensive treatment burden that means a TESE procedure, ovarian stimulation, oocyte retrieval and embryo transfer before starting with the procedures. In addition, they have to consider the emotional impact of a negative result of the TESE procedure, but also the psychological impact of the ICSI treatment if sperm retrieval is successful (Rockliff, et al., 2014, Verhaak, et al., 2007). For counselling we need clinical prediction models which can foresee the chance of successful sperm retrieval in TESE and predict the chance of a live birth adjusted for couple's characteristics. A number of prediction models for successful sperm retrieval in TESE have been developed already but none of them have been externally validated (Boitrelle, et al., 2011, Ramasamy, et al., 2013, Samli and Dogan, 2004, Tsujimura, et al., 2004). In reproductive medicine several prediction models have been developed, but none has been developed and validated for couples requiring TESE-ICSI. It is unknown whether parameters known to be predictive for IVF or ICSI outcome with ejaculated sperm play the same predictive role for these specific couples, as azoospermic couples have no natural chances for pregnancy. It can be imagined that in these couples, different clinical or laboratory parameters will have impact on the probability of getting pregnant and having a live born child.

Paternal ageing and assisted reproductive techniques

In the Netherlands, about ten percent of the couples who are trying to get pregnant are referred to the fertility clinic because of failure to conceive (Nap, 2013). This percentage has increased during time because couples seek for help after a shorter period of exposure but also due to delayed parenthood, second marriages and therefore more infertility cases are found (Nap, 2013). In western society and developed countries, there is a tendency for delayed parenthood as a consequence of social economical welfare, personal education development and increased life expectancy. Not only females are delaying parenthood; male age for parenthood has also increased in the last decade: in the year 2001 in the Netherlands 11.1% (n=22981) of the children born had a father over 40 years old; in 2012 this percentage increased to 16.4% (n=28 888) (CBS, 2011).

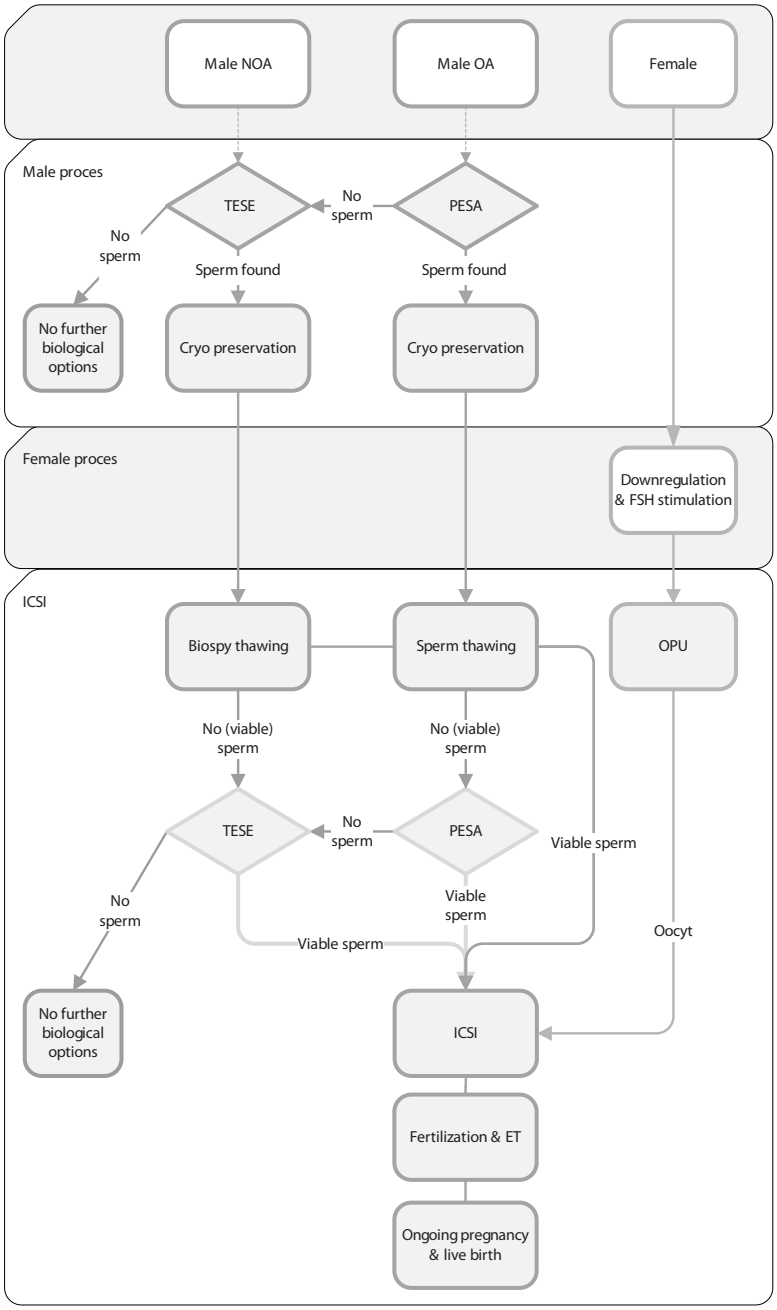

Figure 4 Steps in fertility treatment for couples facing azoospermia

While for female age a biological clock will determine the declining of their fecundity, male's biological clock does not play such a prominent role, as men can produce sperm even at a very advanced age. Assisted reproductive techniques (ART) are commonly used to treat infertile couples. While a maximum access age to ART has been legally determined for women because of the low pregnancy changes and increased genetic risks, such a maximum age not has been considered for men. However, male ageing can compromise spermatogenesis (Plas, et al., 2000), increase DNA methylation (Jenkins, et al., 2013), and increased production of reactive oxygen species can induce DNA damage (Cocuzza, et al., 2008). Moreover, recent epidemiological literature demonstrates an association between paternal age and the prevalence of autism spectrum disorders, schizophrenia and low educational attainment in offspring (D'Onofrio, et al., 2014, Humm and Sakkas, 2013). In addition, Pfeiffer syndrome, Crouzon syndrome, Achondroplasia, Apert syndrome, MEN2A and MEN2B are examples of genetic disorders associated with an advanced paternal age (Goriely and Wilkie, 2012). These disorders have their origin in mutations of paternal imprinted genes FGFR 2, FGFR 3 and/or RET (Crow, 2000) and occur more frequently than expected because of a selective advantage of these mutated cells in spermatogenesis.
Limited or but also controversial results about the influence of paternal age on the reproductive outcome after ART have been published. Previous studies show discordant findings in terms of effects on embryo quality, pregnancy rate and live born delivery (de La Rochebrochard, et al., 2006, Ferreira, et al., 2010, Klonoff-Cohen and Natarajan, 2004). In these studies the use of non-ejaculated sperm (epididymal or testicular origin) was not included. However, particularly elderly males who had a vasectomy are candidates for intracytoplasmic sperm injection (ICSI) with non-ejaculated sperm for ART treatment. Therefore, this subject should be studied more extensively.

Aims and outline of the thesis

This thesis focuses on the safety and efficacy of ART in male infertility and aims to provide tools and information for the physician to give a better counselling to couples facing azoospermia. The following research questions are being studied in this thesis:

- What factors pertain to the prediction of obtaining spermatozoa with TESE in men with NOA and how well does the resulting prognostic model perform using data from another centre? (**Chapter 2**)

- Which parameters have a predictive value for live birth in couples undergoing TESE-ICSI and how well does the resulting prognostic model perform using data from another centre? (**Chapter 3**)

- What is the prevalence of birth defects in newborns born after PESA-ICSI and TESE-ICSI, and is there an effect of maternal and treatment-related factors (lab factors) on ART outcome? (**Chapter 4**)

- Are children born after TESE-ICSI at risk for behavioural, cognitive, motor or physical problems at the age of five? (**Chapter 5**)

- Does paternal age influence the reproductive outcome in ART in terms of the availability of a top quality embryo for transfer, biochemical pregnancy, ongoing pregnancy and miscarriage? (**Chapter 6**)

Chapter 7 provides a general discussion on the findings reported in this thesis, with recommendations for further research.

Chapter 8 is a summary of the thesis.

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Prediction model for obtaining spermatozoa with TESE in men with non-obstructive azoospermia

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Abstract

Study question: Can an externally validated model, based on biological variables, be developed to predict successful sperm retrieval with testicular sperm extraction (TESE) in men with non-obstructive azoospermia (NOA) using a large nationwide cohort?

Summary answer: Our prediction model including six variables was able to make a good distinction between men with a good chance and men with a poor chance of obtaining spermatozoa with TESE.

What is known already: Using ICSI in combination with TESE even men suffering from NOA are able to father their own biological child. Only in approximately half of the patients with NOA can testicular sperm be retrieved successfully. The few models that have been developed to predict the chance of obtaining spermatozoa with TESE were based on small datasets and none of them have been validated externally.

Study design, size, duration: We performed a retrospective nationwide cohort study. Data from 1371 TESE procedures were collected between June 2007 and June 2015 in the two fertility centres.

Participants/materials, setting, methods: All men with NOA undergoing their first TESE procedure as part of a fertility treatment were included. The primary end-point was the presence of one or more spermatozoa (regardless of their motility) in the testicular biopsies. We constructed a model for the prediction of successful sperm retrieval, using univariable and multivariable binary logistic regression analysis and the dataset from one centre. This model was then validated using the dataset from the other centre. The area under the receiver-operating characteristic curve (AUC) was calculated and model calibration was assessed.

Main results and the role of chance: There were 599 (43.7%) successful sperm retrievals after a first TESE procedure. The prediction model, built after multivariable logistic regression analysis, demonstrated that higher male age, higher levels of serum testosterone and lower levels of FSH and LH were predictive for successful sperm retrieval. Diagnosis of idiopathic NOA and the presence of an azoospermia factor c gene deletion were predictive for unsuccessful sperm retrieval. The AUC was 0.69 (95%confidence interval (CI): 0.66–0.72). The difference between the mean observed chance and the mean predicted chance was, <2.0% in all groups, indicating good calibration. In validation, the model had moderate discriminative capacity (AUC 0.65, 95% CI: 0.62–0.72) and moderate calibration: the predicted probability never differed by more than 9.2% of the mean observed probability.

Limitations, reasons for caution: The percentage of men with Klinefelter syndrome among men diagnosed with NOA is expected to be higher than in our study population, which is a potential selection bias. The ability of the sperm retrieved to fertilize an oocyte and produce a live birth was not tested.

Wider implications of the findings: This model can help in clinical decision-making in men with NOA by reliably predicting the chance of obtaining spermatozoa with TESE.

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Introduction

Azoospermia is defined by the complete absence of spermatozoa in the ejaculate (WHO, 2009). It affects ~ 1% of the male population (Jarow, et al., 1989) and can be divided into two main categories; mechanical obstruction along the seminal tract (obstructive azoospermia (OA)) and an intrinsic testicular impairment of sperm production (non-obstructive azoospermia (NOA)). NOA is diagnosed in 60% of azoospermic men (Jarow, et al., 1989, Matsumiya, et al., 1994). Using ICSI in combination with testicular sperm extraction (TESE) even men suffering from NOA are able to father their own biological child.

TESE is an invasive procedure with an, albeit small, risk of complications, including haematoma, inflammation and permanent devascularization, all possibly resulting in the loss of a significant amount of testicular tissue (Donoso, et al., 2007, Schlegel and Su, 1997). Only in approximately half of the patients with NOA can testicular sperm be retrieved successfully (Chan and Schlegel, 2000, Colpi, et al., 2005, Tournaye, 2010).

A number of factors have been suggested to be of predictive value to distinguish patients with a good chance to retrieve sperm cells from patients who have a poor chance. Factors such as testicular volume, serum FSH levels and serum inhibin B levels (Bryson, et al., 2014, Chen, et al., 2010, Toulis, et al., 2010, Yang, et al., 2015, Yildirim, et al., 2014) have been used as variables in several prediction models (Boitrelle, et al., 2011, Ramasamy, et al., 2013, Samli and Dogan, 2004, Tsujimura, et al., 2004). Unfortunately, all these models are of limited value. Most included only a limited number of patients and the models were not validated externally. Since a model tends to perform better in the population in which it has been constructed, external validation is a crucial step before the model can be used in daily practice across different centres (Bleeker, et al., 2003).

It is evident that patients should be well informed about their chances of a successful TESE procedure and their likelihood to retrieve sperm cells before consenting to the procedure. It would therefore be of great value to be able to estimate an individual's chance of sperm retrieval to empower patients in their decision-making. The aim of this study is to develop an externally validated model to predict successful sperm retrieval with TESE in men with NOA in a large nationwide cohort.

Materials and Methods

Study design

We performed a nationwide retrospective cohort study among all men diagnosed with NOA undergoing a first TESE as part of a fertility treatment in the Radboud University Medical Center, Nijmegen (Radboudumc—development set) and the Academic Medical Center, Amsterdam (AMC—validation set), The Netherlands. Data were collected between 1 June 2007 and 1 June 2015. These two centres were the only centres offering TESE–ICSI

during the study period until 1 October 2014, as legal restrictions in the Netherlands limited TESE–ICSI to be conducted only in research settings.

Ethical approval

The protocol for this multicentre study was approved by the Dutch Central Committee on Research involving Human Subjects (NL12408.000.06 CCMO, The Hague, The Netherlands). All couples signed informed consent for treatment and follow-up before participating in this study.

Study population

We included all men diagnosed with NOA and undergoing their first TESE as a part of fertility treatment. NOA was defined as azoospermia without evidence of obstruction and with an elevated level of FSH (>10 UI/l), decreased level of inhibin B (<150 ng/l) and/or small testicular volume (<15 cc per testis) (Adamopoulos and Koukkou, 2010, Jungwirth, et al., 2015). Before surgical sperm extraction, each male underwent a complete andrological evaluation by an urologist. We defined idiopathic NOA when there was no abnormality found in diagnostics such as Klinefelter syndrome, azoospermia factor (AZF)-deletions, cryptorchidism, infection and malignancy. Men with deletions of the AZF-a or AZF-b region of the Y chromosome were excluded for TESE in the Radboudumc. Until September 2010, men diagnosed with Klinefelter syndrome (47XXY) were excluded in both centres due to restrictions in government policies.

TESE procedure

A TESE procedure was performed under local anaesthesia or occasionally under general anaesthesia. A scrotal skin incision was made over the largest testicle or, in case of equal volume, the testicle with the better consistency. Thereafter, the tunica vaginalis was opened and, if necessary, the testis luxated outside the scrotum. The tunica albuginea was longitudinally incised. Either a longitudinal biopsy over the whole length (in case of uniform morphology of the seminiferous tubules) or targeted biopsies of the thicker tubules (in case of differences in quality of the tubules) were taken and immediately transported to the fertility laboratory. The biopsy was then subjected to mechanical dissection and cells present in the lumen of the tubules were extracted (Silber, 2000). The obtained cell suspension was directly examined for the presence of spermatozoa. If no spermatozoa were present in the initial biopsy, then a subsequent biopsy was taken from the contralateral testis. If spermatozoa were present, their number and motility were noted, and the cell suspension was cryopreserved, as described previously (Hessel, et al., 2013).

Outcome

The presence of one or more spermatozoa (regardless of their motility) in the testicular biopsy was considered as successful sperm retrieval and was used as the primary end-point of the study.

Model building

A model was developed to calculate the probability of obtaining sperm with a TESE procedure. Data from Radboudumc were used for building this model (development set). Only the first performed TESE procedure for each couple was included for the analysis. Candidate prognostic parameters or covariates were male age (years), BMI (kg/m2), smoking behaviour (self-reported; yes/no), alcohol consumption (self-reported; yes/no), duration of infertility (months), serum testosterone (nmol/l), inhibin B (ng/l), FSH (IU/l) and LH (IU/l) levels, total testicular volume (cc) (measured by physical examination) and aetiology of NOA (Klinefelter syndrome/AZF-c deletion/cryptorchidism and/or orchidopexy/idiopathic/others).

For each candidate prognosticator, the association with successful sperm retrieval was assessed using thex2 test in a logistic regression model. Collinearity between variables was assessed to prevent the inclusion of redundant variables in the model. All cases were included in the final model, and cases with missing covariate values were imputed using multiple imputation. Missing data varied from 0% to _6% for the potential predictor variables and these values were interpreted as missing at random. We checked the linearity of the association between the continuous variables and a successful sperm retrieval using cubic spline analyses and used transformation in case of non-linearity.

Statistical analysis

Covariates were selected using forward selection (P<0.15 for entry). Backward elimination (P >0.15 for removal) confirmed the covariate selection for the final model. First-order interaction terms and quadratic terms were tested, but not found to be significant.

For the final logistic regression model, we used the Akaike Information Criterion for each imputation set separately to account for differences between sets. Predictors for the final multivariable model were selected using the majority method. The receiver-operating characteristic (ROC) curve was plotted, and the area under the curve (AUC, or c-statistic) was calculated. These characteristics are data driven and presumably too optimistic, therefore the calculated values were denoted as 'apparent' AUC. Optimism corrected values were calculated using leave-one-out cross-validation, i.e. regression coefficients associated with the 'final model' were re-estimated with each case left out in turn. We then combined the 'leave-one-out' regression coefficient with the case's covariate values in order to mimic the prediction of the outcome for each case. Finally, a logistic regression model was fitted with the resulting 'leave-one-out' prognostic index (PI) as the only covariate in order to obtain the optimism-corrected AUC. A histogram displaying the

distribution of the predicted probabilities was plotted. A score chart (Hunault, et al., 2004) was constructed for easier application of the model.

Model validation

External model validation was based on the TESE data from the AMC in Amsterdam (validation set) and focused on two aspects: discrimination and calibration (Leushuis, et al., 2009).

Discrimination is the ability of the model to distinguish between cases with and without the event of interest, in this case between men with successful sperm retrieval with TESE and men where no spermatozoa could be found. Discrimination was measured by the area under the ROC curve, i.e. c-statistic. This statistic ranges from 0.5 (no discrimination) to 1 (perfect discrimination). Calibration refers to correspondence between the predicted probabilities and the observed probabilities. Calibration was assessed visually by comparing predicted probabilities and observed probabilities after dividing men into four groups based on their predicted probability and, more formally, by fitting a logistic regression model with a single covariate for the so-called PI, a linear combination of the case's covariate values and the associated regression coefficients.

For all analyses, we used IBM SPSS Statistics 22 (Chicago, IL, USA) and STATA 14 (Stata-Corp, College Station, TX, USA).

Results

A total of 1371 first TESE procedures were included: 918 in the development set and 453 in the validation set. Baseline characteristics are shown in Table 1. The sperm retrieval rate was 45.6% (419/918) in the development set and was 39.7% (180/453) in the validation set.

Model building

The spline function demonstrated a non-linear association between the continuous variable LH and successful sperm retrieval. We therefore transformed LH to better fit the data using a polynominal: $LH + LH^2$.

Univariable analysis confirmed that older men, men with higher testosterone and high inhibin B levels, men with a history of cryptorchidism and/or orchidopexy and men with a larger testicular volume had significantly higher chances of successful sperm retrieval. Diagnosis of idiopathic NOA, Klinefelter syndrome, detection of an AZF-c deletion and high levels of FSH and LH were significantly associated with lower chances of successful sperm retrieval.

The multivariable logistic regression model (Table 2) included male age, levels of testosterone, LH and FSH, diagnosis of idiopathic NOA and the presence of an AZF-c deletion as independent predictors.

Table 1 Baseline characteristics of men with NOA who underwent a first TESE

		Total (n=1,371)	Development set (n=918)	Validation set (n=453)
Clinical characteristics				
Age (years, SD)		34.29 (6.3)	34.05 (6.02)	34.78 (6.83)
Duration infertility (months, SD)		-	33.36 (25.24)	-
Male BMI (kg/m^2, SD)		-	26.04 (4.13)	-
Smoke n (%)	Yes	-	224 (24.6)	-
	No	-	688 (75.4)	-
Alcohol n (%)	Yes	-	705 (77.5)	-
	No	-	205 (22.5)	-
Testosterone (nmol/L, SD)		13.97 (5.46)	13.43 (5.33)	15.00 (5.55)
LH (IU/L,SD)		8.97 (4.99)	8.57 (4.67)	9.85 (5.53)
FSH (IU/L, SD)		22.13 (12.22)	21.57 (11.83)	23.27 (12.91)
Inhibin B (ng/L, SD)		39.04 (38.85)	40.09 (38.70)	35.89 (39.21)
Total testicular volume (cc, SD)		-	24.94 (9.16)	-
Diagnosis n (%)				
Idiopathic		531 (38.7)	341 (37.1)	190 (41.9)
Klinefelter		85 (6.2)	42 (4.6)	43 (9.5)
AFZ-c deletion		63 (4.6)	35 (3.8)	28 (6.2)
Genetic (others)		12 (0.9)	8 (0.9)	4 (0.9)
Cryptorchidism and/or orchidopexy		450 (32.8)	340 (37.0)	110 (24.3)
Others		209 (15.2)	142 (15.5)	67 (14.8)
Missing		21 (1.5)	10 (1.1)	11 (2.4)

NOA: non-obstructive azoospermia; SD: standard deviation; TESE: testicular sperm extraction, AFZ: azoospermia factor

Cases with missing values for the covariates selected in the final model were evaluated separately. In these 81 cases (FSH (n = 8), LH (n = 46), testosterone (n = 43), AZF deletion (n = 10), idiopathic NOA (n = 10)) the fact of whether data were missing or was not associated with a successful sperm retrieval.

The calculated probability of sperm retrieval in the development set had a range from 6 to 93%, with a mean of 44% (Fig. 1A).

The model had moderate discriminative capacity in the development set. The c-statistic was 0.70 (95% confidence interval (CI): 0.66–0.73) and 0.69 (95% CI: 0.66–0.72) in the optimism-corrected model (Fig. 2A). In the calibration model in the development set, the estimated intercept was 20.01 (95% CI: 20.10 to 0.08) and the slope 1.03 (95% CI: 0.84–1.22). The intercept approached zero and the slope unity. The predicted probability of successful

sperm retrieval was compared with the observed sperm retrieval rate in that category. The difference between the mean observed chance and the mean predicted chance was <2.0% in all groups, which indicates a good calibration of the prediction model in the development set (Table 3 and Fig. 3A).

Table 2 Multivariable logistic regression model for successful sperm retrieval with TESE: stepwise-built logistic model, each row depicting the cumulative contribution of a variable to a model including all variables from previous rows

Covariate	OR	95% CI	p-value	AUC	AUC corrected
Male age	1.06	1.03-1.09	<0.001	0.56	0.56
LH	0.91	0.88-0.93	<0.001	0.63	0.62
LH^2	1.002	1.000-1.003	0.05	0.64	0.63
FSH	0.98	0.96-0.99	0.003	0.65	0.64
Testosterone	1.03	1.01-1.06	0.023	0.65	0.64
AZF-c deletion	0.24	0.11-0.52	<0.001	0.66	0.65
Idiopathic NOA	0.44	0.32-0.59	<0.001	0.69	0.67

AUC: area under the curve; CI: confidence interval; OR: odds ratio.

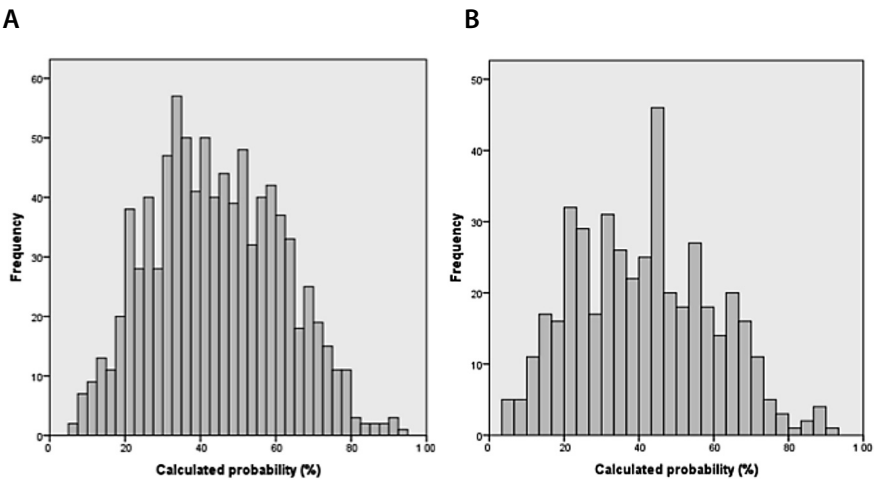


Figure 1 Distribution of the men according to their calculated probabilities of s perm retrieval

A: Development set (n = 918)
B: Validation set (n = 453)

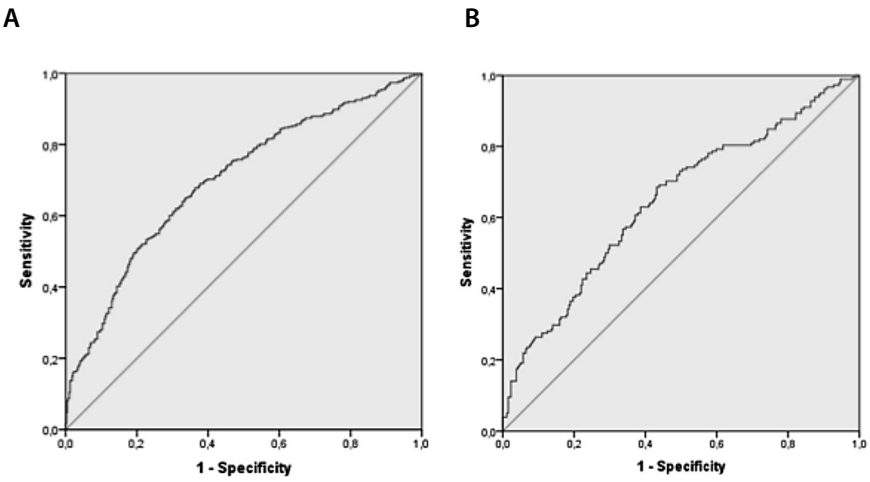


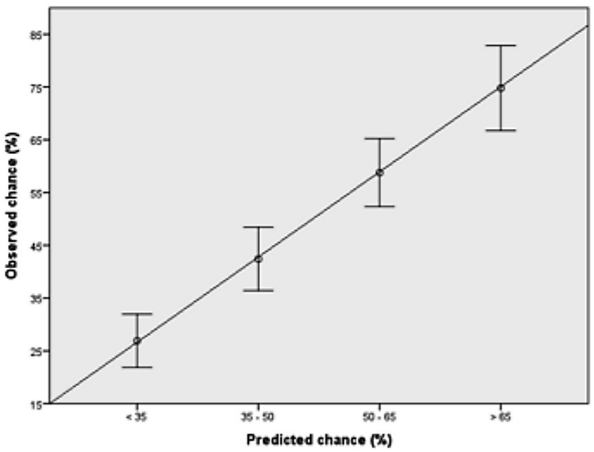
Figure 2 Area under the receiver-operating curve for prediction of sperm retrieval

A: Development set
B: Validation set

Table 3 Mean predicted probability of successful sperm retrieval versus the mean observed successful sperm retrievals

Internal validation				
Predicted chance	No. of patients in group	Mean predicted chance (%)	No. of successful sperm retrievals	Mean observed chance(%)
< 35%	301	25.4	81	26.9
35%-50%	264	42.1	112	42.4
50%-65%	228	57.0	134	58.8
> 65%	115	72.9	86	74.8
External validation				
Predicted chance	No. of patients in group	Mean predicted chance (%)	No. of successful sperm retrievals	Mean observed chance (%)
< 35%	171	23.2	48	28.1
35%-50%	134	42.2	53	39.6
50%-65%	86	57.1	41	47.7
> 65%	51	72.8	36	70.6

A



B

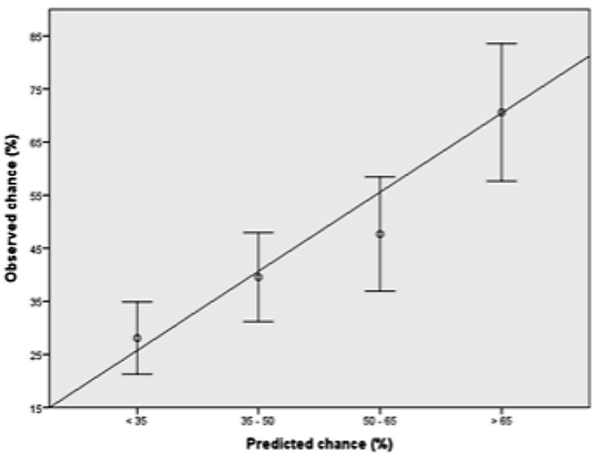


Figure 3 Relationship between calculated and observed sperm retrieval rates.
The four groups represent the quintiles of the calculated probabilities. Data on sperm retrieval rate are reported as percentage and 95% confidence interval

A: Development set
B: Validation set

The mathematical formulation for predicting the probability of sperm retrieval for an individual man is as follows: probability = 1 / [1 + exp (-β)], where β = -1.009 + (male age × 0.058) + (LH ×-0.115) + (LH^2 × 0.001) + (FSH - 0.019) + (testosterone × 0.034) + (AZF-c deletion - 1.480) + (idiopathic NOA - 0.855). Table 4 shows an example of calculated probabilities of successful sperm retrieval with TESE for five hypothetical men based on our prediction model.

External validation

The calculated probabilities of successful sperm retrieval in the validation set ranged from 4 to 92%, with a mean of 41%, indicative of a population with comparable probabilities to the men in the development set (Fig. 1B). The discriminative capacity was in the same range as that in the development set, with a c-statistic of 0.65 (95% CI: 0.59–0.70) (Fig. 2B). The predicted probability of successful sperm retrieval was compared with the observed sperm retrieval rate in that category. The difference between the mean observed chance and the mean predicted chance was 2.2–9.4%, which indicates a moderate calibration of the prediction model (Table 3). Calibration is shown in Fig. 3B. The model showed good calibration below 50% and above 65%. For the predicted sperm retrieval rate between 50 and 65%, a slight overestimation was seen. However, the CIs of the group with a poor predicted chance (<35%) and the group with a moderate predicted change (50–65%) did not overlap, indicating a reliable distinction between these prognostic groups.

Table 4 Five hypothetical men with the calculated probability for obtaining spermatozoa with TESE

	Man A	**Man B**	**Man C**	**Man D**	**Man E**
Age (years)	24	30	34	38	42
LH (IU/L)	6	12	8	6	10
FSH (IU/L)	22	26	20	16	20
Testosterone (nmol/L)	20	10	14	18	12
Aetiology	Klinefelter syndrome	AZF-c deletion	Idiopathic NOA	Cryptorchidism	Idiopathic NOA
Calculated probability for obtaining spermatozoa	50%	11%	34%	70%	39%

Probability = 1/[1+ exp(−β)] where β= -1.009 + (male age x 0.058) + (LH x -0.115) + (LH^2 x 0.001) + (FSH x -0.019) + (testosterone x 0.034) + (AZF-c deletion x -1.480) + (idiopathic NOA x -0.855)

Discussion

This study was designed to develop and validate a model to predict successful testicular sperm retrieval in men with NOA. Our prediction model, built after multivariable logistic regression analysis, demonstrated that higher male age, higher values for serum testosterone and lower values for serum FSH and LH were predictive for successful sperm retrieval. Diagnosis of idiopathic NOA and the detection of an AZF-c deletion were predictive for unsuccessful sperm retrieval. The predictive capacity was moderate with an AUC of 0.69 in the development set and 0.65 in the external validation set. The calibration indicated that our model could distinguish men with a poor prognosis from men with a good prognosis, in terms of sperm retrieval.

A number of prediction models have been developed previously to predict successful sperm retrieval with TESE in men with NOA (Boitrelle, et al., 2011, Ramasamy, et al., 2013, Samli and Dogan, 2004, Tsujimura, et al., 2004). The efficacy of these models was moderate. One study reported a sensitivity of 71.0% and a specificity of 71.4% (Tsujimura, et al., 2004), while another reported a sensitivity of 68.0% and a specificity of 87.5% (Samli and Dogan, 2004). Two studies performed AUC analysis and found an AUC of 0.64 (Ramasamy, et al., 2013) and an AUC of 0.66 (Boitrelle, et al., 2011). None of the developed models was externally validated. For prediction models in the field of infertility, external validation has a demonstrated lower predictive capacity when evaluated in a different population (Altman and Royston, 2000). Thus, these models need external validation before they can be used in clinical practice.

Four of the independent predictors that we included in our final model were also found previously, i.e. male age, and serum FSH, LH and testosterone levels. Furthermore, previously developed prediction models found that testicular volume, duration of infertility, inhibin B, prolactin, Klinefelter syndrome and history of cryptorchidism were independently correlated with TESE outcome. Most of these variables (not prolactin) were included in our univariable analysis and appeared to have no significant correlation with TESE outcome (duration of infertility) or did not retain their significance after multivariable analysis (testicular volume, inhibin B, Klinefelter syndrome and history of cryptorchidism).

This study has some limitations that need to be addressed. First, there is a potential selection bias, due to the restrictions in government policies on men diagnosed with Klinefelter syndrome. Until September 2010, these men were excluded from undergoing TESE in the Netherlands. The percentage of men with Klinefelter syndrome among men diagnosed with NOA is expected to be higher (11%; (Aksglaede and Juul, 2013)) than in our study population (4.6% in the development set and 9.5% in the validation set). Secondly, there may be some confounders that affect the chances of finding spermatozoa, such as patient selection, and clinical and laboratory techniques (Vloeberghs, et al., 2015). There are no rigorously designed randomized studies that compare the various surgical techniques for effectiveness and safety. A recent meta-analysis, which found that the

sperm retrieval rate is higher for micro-TESE compared with conventional TESE and for conventional TESE compared with testicular sperm aspiration (Bernie, et al., 2015), also had some serious limitations. The heterogeneity of the population of men diagnosed with NOA was high and consideration of other variables, such as tissue processing techniques, was lacking. Moreover, the factors that we have identified as predictors in sperm retrieval by TESE are likely to be predictive for any of the techniques used to retrieve spermatozoa from the testis. Finally, we did not measure prolactin levels in our study population on a routine basis although it has been mentioned as a possible prognosticator. Of note, previous studies found no differences between prolactin levels in men with a successful sperm retrieval compared with men with unsuccessful sperm retrieval, rendering it unlikely that prolactin is a main predictor for testicular sperm retrieval (Samli and Dogan, 2004, Tsujimura, et al., 2004).

A strength of our nationwide cohort is the large number of men that we studied. With a total of 1371 first TESE procedures, it is to our knowledge the largest study on this subject reported thus far. The performance of the external validation of our model further strengthens our study. This study shows that our prediction model can be applied in a general population of men with NOA, without losing its accuracy, regardless of the setting of the hospital.

Once TESE is successful, the second step in achieving a pregnancy is ICSI with the surgically retrieved spermatozoa. Again, predicting the chance of success (in this case live birth) is then important to allow informed decisions to be made. Two studies have attempted to find predictive factors for pregnancy outcome after TESE (Silber, et al., 1997, Vernaeve, et al., 2004). We recently developed a prediction model where we address the clinical decision to start or continue with ICSI using TESE derived spermatozoa before treatment or after one or more unsuccessful ICSI treatments (Meijerink, et al., 2016). This model is built using the same cohort of patients as this study, with the obvious exception that only those couples that initially underwent a successful sperm recovery with TESE are included in the prediction model for live birth. The model has a moderate discriminative capacity (AUC of 0.62 in the development set; AUC of 0.67 in the validation set) and calibrates well. Appropriate counselling of men undergoing TESE is an important issue from a psychological viewpoint. Using this model it is possible to counsel men individually about their chance of successfully obtaining spermatozoa with TESE. Considering their individual chance to obtain spermatozoa together with their personal options and preferences will help men and their partners to decide whether to undergo a TESE procedure or not. Men with a poor prognosis might decide to refrain from this treatment option and reconsider artificial insemination of their partner with donor sperm, adoption and/or becoming foster parents to fulfil the child wish. Future studies should clarify patients' preferences and allow clinicians to provide shared decision-making (den Breejen, et al., 2013).

In conclusion, this study demonstrates that in the prediction of successful testicular sperm retrieval a distinction can be made between men with a good prognosis and men with a

poor prognosis. The success of sperm retrieval was found to depend on male age, FSH, LH, and testosterone levels, diagnosis of idiopathic NOA and detection of an AZF-c deletion. After external validation, the model proved to be accurate in predicting chances of success with TESE. We propose that our model should be used for counselling men with NOA.

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3

Prediction model for live birth in intracytoplasmic sperm injection using testicular extracted sperm

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Abstract

Study question: Which parameters have a predictive value for live birth in couples undergoing ICSI after successful testicular sperm extraction (TESE-ICSI)?

Summary answer: Female age, a first or subsequent started TESE-ICSI cycle, male LH, male testosterone, motility of the spermatozoa during the ICSI procedure and the initial male diagnosis before performing TESE were identified as relevant and independent parameters for live birth after TESE-ICSI.

What is known already: In reproductive medicine prediction models are used frequently to predict treatment success, but no prediction model currently exists for live birth after TESE-ICSI.

Study design, size, duration: A retrospective cohort study between 2007 and 2015 in two academic hospitals including 1559 TESE-ICSI cycles. The prediction model was developed using data from one centre and validation was performed with data from the second centre.

Participants/materials, setting, methods: We included couples undergoing ICSI treatment with surgically retrieved sperm from the testis for the first time. In the development set we included 526 couples undergoing 1006 TESE-ICSI cycles. In the validation set we included 289 couples undergoing 553 TESE-ICSI cycles. Multivariable logistic regression models were constructed in a stepwise fashion
($P < 0.2$ for entry). The external validation was based on discrimination and calibration.

Main results and the role of chance: We included 224 couples (22.3%) with a live birth in the development set. The occurrence of a live birth was associated with lower female age, first TESE-ICSI cycle, lower male LH, higher male testosterone, the use of motile spermatozoa for ICSI and having obstructive azoospermia as an initial suspected diagnosis. The area under the receiver operating characteristic (ROC) curve was 0.62. From validation data, the model had moderate discriminative capacity (c-statistic 0.67, 95% confidence interval: 0.62–0.72) but calibrated well, with a range from 0.06 to 0.56 in calculated probabilities.

Limitations, reasons for caution: We had a lack of data about the motility of spermatozoa during TESE, therefore, we used motility of the spermatozoa used for ICSI after freeze-thawing, information which is only available during treatment. We had to exclude data on paternal BMI in the model because too many missing values in the validation data hindered testing. We did not include a histologic diagnosis, which would have made our data set less heterogeneous and, finally, our model may not be applicable in centres which have a different policy for the indication for performing sperm extraction. The prognostic value of the model is limited because of a low 'area under the curve'.

Wider implications of the findings: This model enables the differentiation between couples with a low or high chance to reach a live birth using TESE-ICSI. As such it can aid in the counselling of patients and in clinical decision-making.

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Introduction

One revolutionary achievement in assisted reproductive techniques (ART) has been the introduction of ICSI in 1992 (Palermo, et al., 1992). ICSI allowed couples with severe oligo-zoospermia to father their genetically own offspring. Subsequently, ICSI was used in conjunction with testicular sperm extraction (TESE) in men with azoospermia (Devroey, et al., 1995).

Spermatozoa are found in _50% of the men with non-obstructive azoospermia (NOA) who undergo TESE (Chan and Schlegel, 2000, Tournaye, et al., 1995). Surgical sperm retrieval carries a small but existing complication risk, such as loss of significant amounts of testicular tissue, haematoma, inflammatory changes and permanent devascularisation (Schlegel and Su, 1997). Only one out of seven men undergoing TESE eventually fathers a genetically own child (Vloeberghs, et al., 2015). We recently developed and externally validated a prediction model for obtaining spermatozoa with TESE in men with NOA (Cissen, et al., 2016).

Once spermatozoa are successfully retrieved, it is possible to start ICSI treatment. Although TESE-ICSI is a blessing for the NOA couple, it is in our opinion still important to inform patients of their realistic chances to conceive. Couples should have the possibility to consider whether it is worth the intense treatment burden that entails ovarian stimulation, oocyte retrieval, and not to forget the emotional impact of a failed treatment (Rockliff, et al., 2014, Verhaak, et al., 2007). For this we need a clinical prediction model which can predict the chance of a live birth adjusted for couple's characteristics.

In reproductive medicine several prediction models have been developed for spontaneous pregnancy, pregnancy after intrauterine insemination and pregnancy after IVF/ICSI (Leushuis, et al., 2009). In studies that predict the outcome of ICSI, it was demonstrated that maternal age is the most important predictive parameter next to the duration of infertility and obstetric history (Lintsen, et al., 2007, Stolwijk, et al., 2000). Nevertheless, it is unknown if these parameters play the same predictive role for couples who require TESE-ICSI, as these couples have no natural chances for pregnancy. It is possible that in these specific couples, other parameters will have an impact on the probability of getting pregnant.

The aim of this study was to develop and validate a model for couples who had a successful TESE, to predict their chance of having a live birth with TESE-ICSI.

Materials and Methods

Study design

Between 1 September 2007 and 1 May 2014 we performed a retrospective cohort study, among couples undergoing TESE-ICSI at the Radboud university medical center, The Netherlands (Radboudumc). The data we collected from Radboudumc were used to develop a model to predict live birth after a TESE-ICSI cycle (development set). An ICSI cycle was defined as a fresh cycle and the corresponding cryo embryo cycle(s) derived from it; each cycle was considered as a separate unit of analysis. Between 1 August 2007 and 1 September 2015, we collected data for validation of the model in couples undergoing TESE-ICSI in the Academic Medical Center (AMC), The Netherlands (validation set).

Ethical approval

Until 2014 in the Netherlands, a TESE procedure was allowed only in research settings. The protocol for this study was approved by the Dutch Central Committee on Research involving Human Subjects (NL12408.000.06CCMO, The Hague, The Netherlands). All couples signed an informed consent for treatment and follow-up before participating in this study.

Study population

The study population contained couples eligible for ICSI treatment. We included men with azoospermia—defined as no spermatozoa found in the sediment of a centrifuged sample, confirmed in at least two semen analyses (WHO, 2009)—who, after a complete andrologic evaluation by an urologist, were diagnosed with NOA and subsequently underwent a successful TESE procedure or men who were initially suspected for obstructive azoospermia (OA), underwent an unsuccessful percutaneous epididymal sperm aspiration (PESA) procedure, but a successful TESE procedure, i.e. viable spermatozoa were found. The TESE procedure was performed in both clinics. We excluded men with deletions in the AZF-a or AZF-b region of the Y chromosome and Klinefelter syndrome.

TESE procedure

All TESE procedures were performed by a trained urologist. The description of the local clinical protocol has been published (Hessel, et al., 2013). In summary, a conventional longitudinal testicular biopsy according to the method described by Silber was performed in all men (Silber, 2010) before the start of female hormonal stimulation, to make sure that viable sperm were available at oocyte retrieval. If spermatozoa were found in the testicular biopsy, aliquots of sperm cell suspensions were cryopreserved and female hormonal stimulation was started and the sperm cell suspension was thawed at oocyte retrieval. In case no viable sperm was found, a second TESE was performed with the agreement of the patient, and in the absence of physical or medical contraindication, and fresh sperm cells

were used at oocyte retrieval. Surplus aliquots of spermatozoa cell suspensions of the second TESE were cryopreserved as well.

ICSI cycles

All couples underwent at least one ICSI cycles. Controlled ovarian hyperstimulation was performed with recombinant FSH (Puregon or Gonal-F) or human urinary FSH (Menopur or Fostimon) after pituitary down-regulation with a GnRH agonist or antagonist. Oocyte retrieval took place by ultrasound-guided needle aspiration, 34–36 h after hCG (5000 or 10 000 IU) administration. Only metaphase II, morphologically normal oocytes were injected with frozen–thawed or fresh spermatozoa. In case of immotile spermatozoa, viable spermatozoa were selected using the tail touch procedure (de Oliveira, et al., 2004, Hessel, et al., 2015). The tail touch procedure involves the recording of sperm tail flexibility after 2 h of incubation. The tail of the spermatozoon is gently touched with an ICSI micropipette, and when the tail is flexible the spermatozoon is considered viable.

Embryo scoring and evaluation for transfer was based on fragmentation and the number of blastomeres, assessed by a qualified embryologist or laboratory technician using a light inverted microscope, at standard set points: fertilization, early cleavage stage, Day 2 and Day 3 of development (ESHRE, 2000).

Intrauterine embryo transfer was performed at the third, fourth or fifth day after oocyte retrieval. Single embryo transfer (SET) or double embryo transfer (DET) was performed depending on female age, female medical history, the couple's preference and national policy. National embryo transfer policy changed over time, which brought about compulsory SET in women <38 years in the first two cycles for the last 3 years of the study period. No embryo transfer was performed in case of absence of fertilization, abnormal embryos or in case of (risk for) ovarian hyperstimulation syndrome (OHSS).

Luteal phase was supported by vaginal administration of 600 mg progesterone per day. An ongoing pregnancy was defined as the appearance of a foetal heartbeat examined by ultrasound after 12 weeks of gestational age. Live birth was defined as the complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or shows any other evidence of life such as heart beat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached (Zegers-Hochschild, et al., 2009).

Building the predictive model

We used live birth as the primary end-point of the study. We defined one delivery as the birth of one singleton baby or multiples. We developed a model by calculating the probability of live birth after a TESE-ICSI cycle. We considered each started cycle including corresponding cryo embryo cycle(s) as a separate unit of analysis. Model building was based on TESE-ICSI data from the development set (Radboudumc).

Based on the current literature, previous prediction models and our own expectations, we identified a number of candidate predictors (Boitrelle, et al., 2011, Friedler, et al., 2002, Leushuis, et al., 2009, Ramasamy, et al., 2013, Tehraninejad, et al., 2012, van Loendersloot, et al., 2013). The candidate baseline parameters/covariates were as follows:

Type of infertility (primary/secondary);
Duration of infertility (months);
Female age (years);
Parity (n);
Average menstrual cycle length (days);
Uterine abnormalities (yes/no);
Antral follicle count before stimulation (number of follicles <11 mm);
Alcohol use (self-reported; yes/no) for male and female;
Smoking status (self-reported; yes/no) for male and female;
BMI at baseline (kg/m^2) for male and female;
Male age (years);
Male testosterone (nmol/l);
Male inhibin B (ng/l);
Male FSH (IU/l);
Male LH (IU/l);
Total testicular volume (cc);
Suspected primarily diagnosis of azoospermia (OA/NOA) before sperm retrieval.

NOA was defined as azoospermia, in combination with either small testes (volume per testis <15 ml), elevated level of FSH (>10 IU/l) and/or decreased level of inhibin B (<150 ng/l) (Adamopoulos and Koukkou, 2010, Jungwirth, et al., 2012) and without evidence of obstruction. In men with evidence of obstruction and with normal testes volume, FSH and inhibin B levels, the initial suspected diagnosis was OA.

The candidate cycle parameters/covariates were:
Number of TESE-ICSI cycles;
Spermatozoa (fresh or frozen–thawed);
Motility of spermatozoa (oocytes injected with motile spermatozoa/immotile spermatozoa or a combination of both for each individual cycle);
Number of oocytes retrieved.
Ideally, all parameters in a prognostic model are available before start of the treatment. In this case it would be preferable to include the motility of spermatozoa found at the TESE procedure. However, due to a lack of these data we decided to use the motility of the spermatozoa used for ICSI (in most cases after freeze-thawing). Besides motility, three other cycle depended parameters were also analysed as described above.

Statistical analysis

For each candidate prognostic variable, the association with occurrence of an ongoing pregnancy leading to a live birth was assessed using the χ score test in a logistic regression model. Colinearity between variables was assessed to prevent the inclusion of redundant variables in the model. After the inclusion of female age, covariates were selected using forward selection ($P < 0.20$ for entry). Backward elimination ($P > 0.20$ for removal) confirmed the covariate selection for the final model. All subjects were included in the final models with missing covariate values imputed using linear regression. First-order interaction terms and quadratic terms were tested, but not found to be statistically significant.

We first analysed our data with generalized estimating equations (GEE) and afterwards with logistic regression. The point estimates and confidence intervals (CI) after analysis with GEE were almost identical to those of logistic regression. As logistic models are easier to interpret and the point estimates did not differ, we decide to use multivariable logistic regression to develop a model.

For the final logistic regression model the receiver operating characteristic (ROC) curve was plotted and the area under the curve (AUC, or c-statistic) was calculated.

As the capacity of a variable to predict live birth may vary in a series of ICSI cycles, we explicitly tested statistically for interactions between included predictors and ICSI cycle number. In deciding between competing expressions of related parameters, we used Akaike's information criterion in variable selection.

These characteristics are data driven and presumably too optimistic. Optimism-corrected values were calculated using leave-one-out crossvalidation, i.e. the regression coefficients associated with the 'final model' were re-estimated with each subject left out in turn. We then combined the 'leave-one-out' regression coefficient with the subject's covariate values in order to mimic the prediction of the outcome for each subject. Finally, a logistic regression model was fitted with the resulting 'leave-one-out' prognostic index as the only covariate in order to obtain the optimism-corrected AUC. A histogram (not shown) displaying the distribution of the predicted probabilities was plotted.

Model validation

A crucial aspect of a prediction model derived from one data set is the wider applicability to a data set from another centre or from a different time period. The idea of validating a prognostic model is generally taken to mean establishing that it works satisfactorily for patients other than those from whose data the model was derived (Altman and Royston, 2000). External model validation was based on the TESE-ICSI data from the validation set (at AMC) and focused on two aspects: discrimination and calibration (Leushuis, et al., 2009).

Discrimination was measured by the area under the ROC curve, the c-statistic. This statistic ranges from 0.5 (no discrimination) to 1 (perfect discrimination). Calibration refers to

correspondence between the predicted probabilities and the observed proportions. Calibration was assessed visually by comparing predicted probabilities and observed proportions after dividing patients in six groups based on their predicted probability and, more formally, by fitting a logistic regression model. All analyses were performed using IBM SPSS Statistics 22 (Chicago, IL, USA) and STATA 14 (Texas, USA).

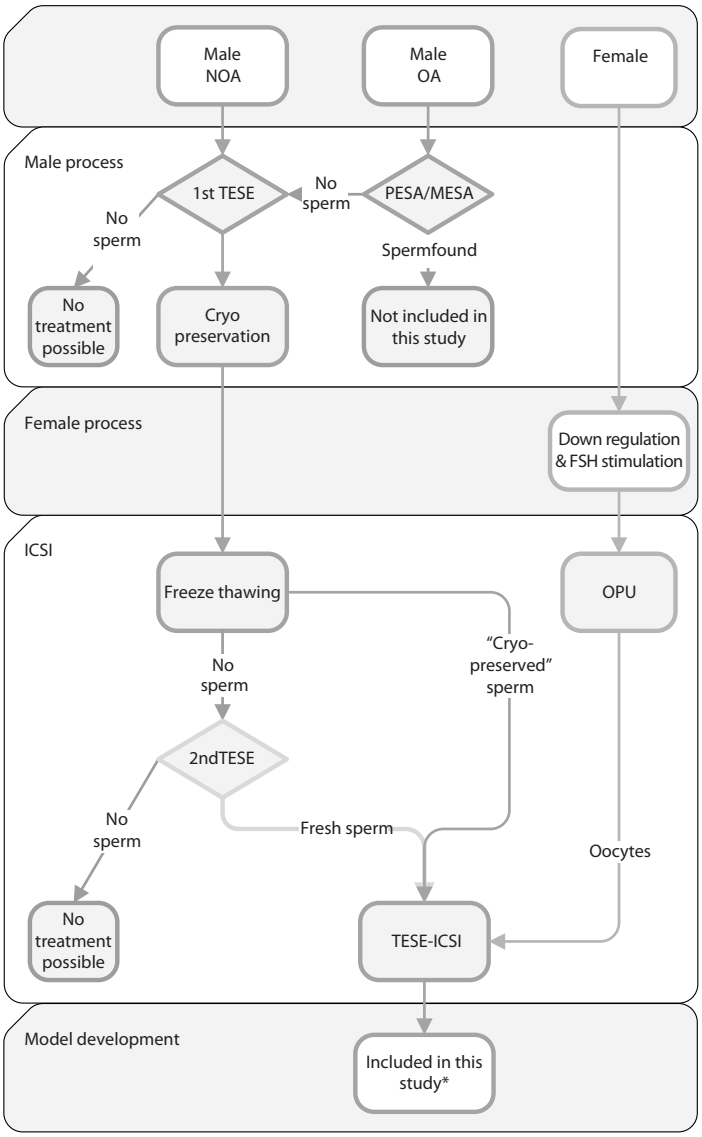
Results

In this study we included in total 815 couples who had undergone 1559 TESE-ICSI cycles for the development and validation set. Figure 1 shows the steps to take for a couple undergoing TESE-ICSI treatment and Table 1 shows the baseline characteristics. For the development set, we included 526 couples who had undergone 1006 TESE-ICSI cycles of which 379 men (72.1%) were diagnosed with NOA and 147 men (27.9%) with suspected OA but with no sperm retrieval after PESA or microsurgical epididymal sperm aspiration (MESA) and successful testicular sperm retrieval (OA.NOA diagnosis group). In 73 couples (80 cycles) there was no embryo transfer: 66 cycles resulted in a total failure of fertilization, in 10 cycles there was no suitable embryo for transfer present and in 4 cycles a fresh embryo transfer was withheld because of imminent OHSS. The cumulative ongoing pregnancy rate per cyclewas 229 (22.8%) of which 224 (22.3%) resulted in a live birth of 1 or more children: 196 singletons, 27 twins and 1 triplet. Three pregnancies were terminated because of congenital anomalies: 1 with both a hygroma colli and an omphalocele, 1 with trisomy 18 and 1 because of craniocephalic malformation. Two pregnancies involved a stillbirth at 20 and 41 weeks of gestational age, respectively.

For the external validation set, we included 289 couples who had undergone 553 TESE-ICSI cycles of which 169 men (58.4%) were diagnosed with NOA and 105 men (36.3%) were in the OA>NOA diagnosis group. In 15 men (5.2%) the initial diagnosis was unclear due to missing hormonal values. There were 119 ongoing pregnancies (21.5%) of which 113 (20.4%) resulted in a live birth of 1 or more children. Univariable analysis showed that younger women, younger men, men with lower LH, lower FSH and higher testosterone levels, and those with motile spermatozoa used for ICSI and having an OA as initial diagnosis and those undergoing the first ICSI cycle, had significantly higher chances of a live birth. None of the hormone values were below the limit of detection.

We included six predictors in the final multivariable logistic regression model: female age, cycle number, male LH level, male testosterone level, sperm motility and suspected diagnosis before sperm retrieval (OA versus NOA). When we added a square term for female age, we did not find interactions, nor did interaction terms improve the model.

In the development set three variables we had selected for the final model, were incomplete, i.e. male LH (9%), male testosterone (9%) and sperm motility (1%) had missing values. In the validation set four variables we had selected were incomplete, i.e. male LH



Suspected diagnosis based on medical history, physical examination and hormonal values. NOA: Non obstructieve azoospermia; OA: obstructive azoospermia; TESE: testicular sperm extraction; PESA: percutaneous epididymal sperm aspiration; MESA: microsurgical epididymal sperm aspiration; FSH: follicular stimulating hormone; ICSI: intracytoplasmic sperm injection; OPU: ovum pick up.

* Following cycles of couples who already had aprevious live birth after TESExICSI during the study period were excluded.

Figure 1 Steps in TESE-ICSI treatment

Table 1 Baseline characteristics of testicular sperm extraction (TESE)-ICSI cycles included in this study

	Total n=1559	**Development set n=1006**	**Validation set n=553**
No. of couples (n)	815	526	289
Clinical characteristics mean (±SD)			
Female age years	32.8 (4.5)	32.4 (4.4)	33.7 (4.5)
Male age years	38.5 (8.5)	38.2 (8.1)	39.1 (9.0)
Type of infertility per cycle			
Primary infertility n (%)		830 (82.5)	
Secondary infertility n (%)		176 (17.5)	
Duration of infertility months mean (±SD)		41.5 (25.2)	
Female parameters per cycle n (%)			
Endometriosis		18 (1.8)	
Polycystic ovary syndrome		50 (5.0)	
Congenital uterine anomaly		4 (4.0)	
Acquired uterine anomaly		13 (12.9)	
Male hormones at baseline mean (±SD)			
Male testosterone nmol/l	15.2 (5.8)	14.8 (6.0)	16.0 (5.4)
Male inhibin B ng/l		92.2 (79)	
Male FSH IU/l	13.9 (11.6)	13.6 (11.3)	15.0 (12.8)
Male LH IU/l	6.4 (4.1)	6.0 (3.6)	7.5 (5.0)
No. ICSI cycles n (%)			
1st cycle	815 (52.3)	526 (52.3)	289 (52.3)
2nd cycle	466 (29.9)	306 (30.4)	160 (28.9)
3th cycle	222 (14.2)	137 (13.6)	85 (15.4)
≥4th cycle	56 (3.6)	37 (3.7)	19 (3.4)
Sperm characteristics			
Sperm condition used in ICSI cycle n (%)			
Fresh	239 (15.3)	200 (19.9)	39 (7.1)
Frozen thawed	1317 (84.5)	806 (80.1)	511 (92.4)
Unknown	3 (0.2)	0	3 (0.5)
Sperm motility used in ICSI cycle n (%)			
Motile	773 (49.6)	641 (63.7)	132 (15.7)
Immotile	180 (11.5)	138 (13.7)	42 (7.6)
Both motile & immotile	305 (19.6)	218 (21.7)	87 (23.9)
Motility unknown	301 (19.3)	9 (0.9)	292 (52.8)

Table 1 Continued

	Total n=1559	Development set n=1006	Validation set n=553
Laboratory data per cycle mean (±SD)			
No. oocytes	10.5 (5.6)	10.3 (5.2)	10.9 (6.2)
No. oocytes fertilized		4.4 (3.1)	
No. 2pn embryos	4.1 (3.2)	4.3 (3.0)	3.8 (3.4)
No. frozen embryos		0.6 (1.3)	
No. embryos transferred	1.3 (0.7)	1.4 (0.6)	1.2 (0.8)
Pregnancies n (%)			
No. ongoing pregnancies	348 (22.3)	229 (22.8)	119 (21.5)
No. live births	337 (21.6)	224 (22.3)	113 (20.4)

No.: number; Acquired uterine anomaly: uterus myomatosus or endometrial polyp; PN: pronuclei

(9%), male testosterone (3%), sperm motility (53%) and initial diagnosis (4%) had missing values. Whether or not data were missing was not associated with the occurrence of an ongoing pregnancy leading to a live birth and were not significant in the analysis described above.

The multivariable analysis is presented in Table 2. We did not find a significant additional effect of ICSI cycle number, nor did we find any significant interactions between the identified predictors and cycle number. For this reason, we used the same point estimates for all predictors and we included cycle number as a predictor.

Table 2 Multivariable analysis for predicting live birth after a TESE-ICSI cycle

Predictors	OR	95% CI	*p-value*	*AUC*	*AUC corrected*
Female age^2	0.99	0.98-0.99	0.02	0.56	0.56
No. of ICSI cycles	0.76	0.56-1.04	0.07	0.58	0.58
Male LH	0.94	0.98-0.99	0.02	0.62	0.62
Male testosterone	1.03	1.00-1.05	0.06	0.61	0.61
Sperm motility	1.82	1.09-3.04	0.02	0.60	0.60
NOA vs. OA>NOA	1.31	0.90-1.97	0.09	0.63	0.63

Suspected diagnosis before sperm retrieval based on medical history, physical examination and hormonal values. OA>NOA: suspected OA but with no sperm retrieval after epididymal sperm aspiration.

OR: Odds ratio; CI: confidence interval; AUC: area under the curve; Female age^2: age square; NOA: non-obstructive azoospermia; OA: obstructive azoospermia.

The calculated probabilities of a live birth for the 526 couples in the development set ranged from 0.03 to 0.47, with a mean of 0.19. Twenty-five per cent of the TESE-ICSI cycles had a probability of a pregnancy less than 0.14, 25% had a probability between 0.14 and 0.19, 25% a probability between 0.19 and 0.24, and 25% had a probability exceeding 0.24. In the development set the model had moderate discriminative capacity with a c-statistic of 0.63 (95% CI: 0.59–0.67) and in the over optimism corrected model 0.62 (95% CI: 0.58–0.68) (Fig. 2). The model calibrated well; the goodness-of-fit test (Hosmer–Lemeshow) showed no significant miscalibration (P= 0.79). Figure 3A shows the calibration plot in the development set. In the case of perfect calibration, all points would be on the diagonal, the line of equality and average probabilities correspond perfectly to the observed live birth rates. Our calibration plot showed that the model calibrated well. In the calibration model in the development set, the estimated intercept was 0.02 (95% CI: 20.05 to 0.09) and the slope 1.07 (95% CI: 0.89–1.25). The intercept approached zero and the slope unity.

External validation

External model validation was based on the TESE data from the AMC in Amsterdam (validation set) and focused on two aspects: discrimination and calibration (Leushuis, et al., 2009).

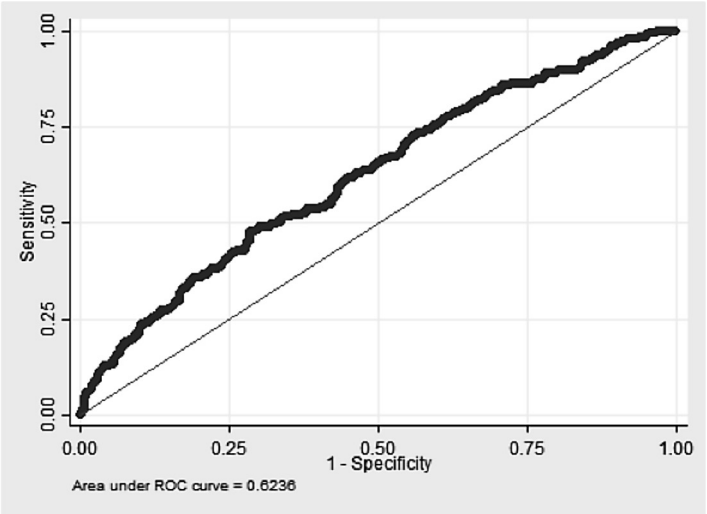
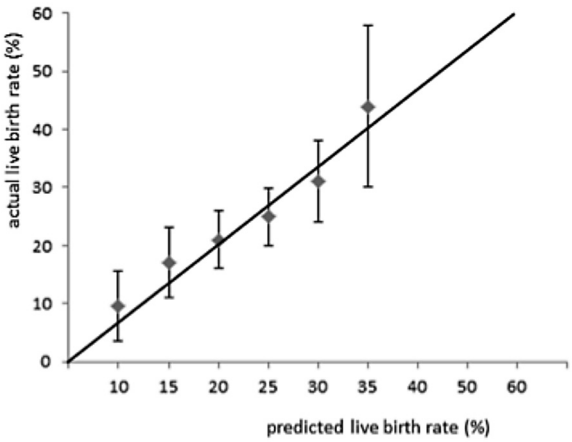


Figure 2 Receiver operating characteristic curve (ROC) of the multivariable logistic regression model for the prediction of live birth after TESE-ICSI (model development)

Discrimination is the ability of the model to distinguish between cases with and without the event of interest, in this case between men with successful sperm retrieval with TESE and men where no spermatozoa could be found. Discrimination was measured by the area under the ROC curve, i.e. c-statistic. This statistic ranges from 0.5 (no discrimination) to 1 (perfect discrimination). Calibration refers to correspondence between the predicted probabilities and the observed probabilities. In the external validation set the model had slightly better discriminative capacity. The c-statistic was 0.67 (95% CI: 0.62–0.72) (Fig. 4). The model calibrated well; the goodness-of-fit test (Hosmer–Lemeshow) showed no significant miscalibration (P = 0.73). The calibration plot visually resembles the calibration

A



B

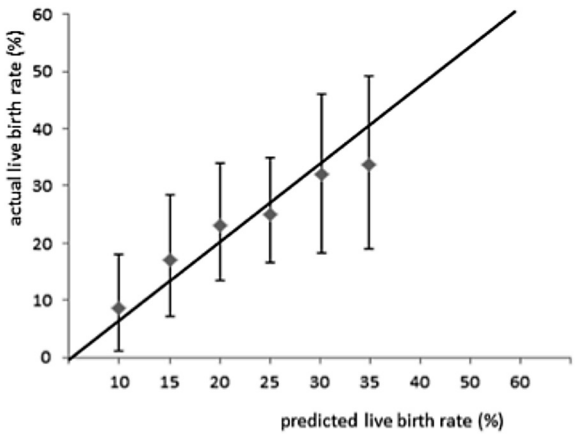


Figure 3 Calibration plots with calculated probability on the X-axis and observed proportion on Y-axis, development set (**A**) & validation set (**B**)

of the development set though with larger 95% boundaries and a range from 0.06 to 0.56 in calculated probabilities (Fig. 3B).

Table 3 presents the prediction model and shows an example of calculated probabilities of a live birth in a TESE-ICSI cycle for five hypothetical couples based on our prediction model. It should be taken into account whether or not the spermatozoa used for ICSI are motile. For example couple C of which the female age is 34 years, who will have their first ICSI cycle, with a male LH of 4 IU/l and a male testosterone of 20 nmol/l in a man diagnosed with NOA, the chance of a live birth in the first ICSI cycle is 22% when there are enough motile spermatozoa for the injection of all oocytes and 13% when there are not.

Validation of the model without sperm motility

The sperm motility was only known for 261 (47%) cycles of the external validation set therefore we also made a model without sperm motility. This model, including all previously selected variables while excluding sperm motility, had a c-statistic of 0.61 (95% CI: 0.57–0.65) in the development set and 0.62 (95% CI: 0.55–0.69) in the validation set. However this model did not calibrate well and therefore this model is not discussed further.

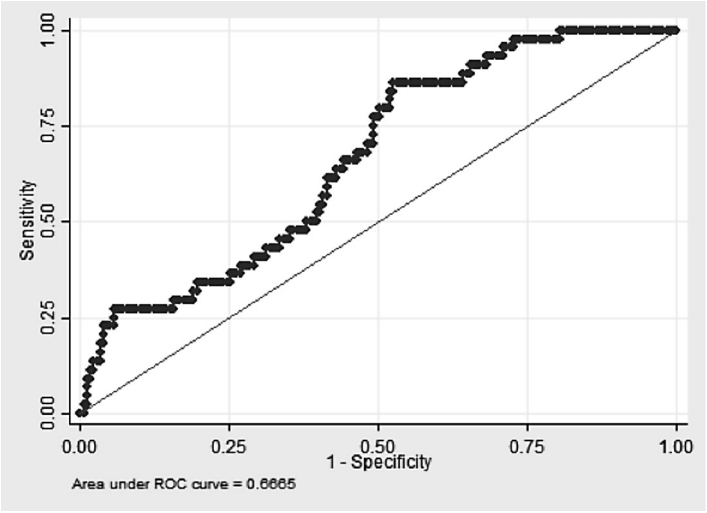


Figure 4 Receiver operating characteristic curve of the multivariable logistic regression model for the prediction of live birth after TESE-ICSI (model validation)

Table 3 Five hypothetical couples with the calculated probability of a live birth in a TESE-ICSI cycle

	Couple A		Couple B		Couple C		Couple D		Couple E	
Female age (years)	24		30		34		38		40	
ICSI cycle	1		2		1		2		1	
Male LH (E/L)	4		8		4		10		6	
Male Testosterone (nmol/L)	20		14		20		10		14	
NOA(=0) or OA>NOA (=1)	OA>NOA		NOA		NOA		NOA		OA>NOA	
Motile spermatozoa for ICSI Yes (=1) or No (=0)	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Calculated probability of a live birth per cycle	0.31	0.20	0.16	0.09	0.22	0.13	0.06	0.04	0.10	0.06

Probability of live birth after a TESE-ICSI cycle= EXP (-7.659 + 0.442*Female age - 0.008*Female age^2 - 0.278 *No. ICSI cycle + 0.608*Sperm motility + 0.026*Testosterone - 0.06*LH + 0.269*(N)OA) / (1 + EXP (-7.659 + 0.442*Female age - 0.008*Female age^2 - 0.278*No. ICSI cycle + 0.608*Sperm motility + 0.026*Testosterone -0.06*LH + 0.269*(N)OA)).

Suspected diagnosis before sperm retrieval based on medical history, physical examination and hormonal values. OA>NOA: suspected OA but with no sperm retrieval after epididymal sperm aspiration.

Discussion

In our analysis of 526 infertile couples suffering from azoospermia who had 1006 TESE-ICSI cycles, we found that a live birth was associated with a lower female age, the first versus subsequent TESE-ICSI cycles, lower male LH, higher male testosterone, availability of motile spermatozoa for ICSI and having OA as an initial diagnosis. After model development using multivariable logistic regression analysis, we found an AUC of 0.62, and good calibration. We validated our model externally with 553 cycles in 289 infertile couples in a second academic hospital performing TESE-ICSI during the same study period. In the external validation set the model had a slightly better discriminative capacity (AUC 0.67) and calibrated well.

We found similar predictors, such as female age and the number of started cycles (first versus subsequent), as previous studies on IVF/ ICSI had found (Lintsen, et al., 2007, van Loendersloot, et al., 2013). These models are however not applicable to our couples since the females are not per se infertile and the azoospermic men carry specific predictors, not to be found in men with oligo or normospermia.

We found that a lower male LH and a higher male testosterone level are of predictive value for live birth after TESE-ICSI in our study group. Since the production of testosterone in the Leydig cells is regulated by LH, a lower male LH will indicate a (relative) normal testicular function because of negative feedback by testosterone on LH. In men with NOA the hypothalamic-pituitary axis is often already deregulated leading to low testosterone levels, but obesity might also contribute to low testosterone. Unfortunately, we could not include paternal BMI as a predictor in our model, because of too many missing values. Perhaps, if we had complete data, paternal BMI might play a minor predictive role. One previous study found that sperm retrieval rates were similar in normal weight and obese men, but a lower male BMI predicted a higher chance of clinical pregnancy after TESE-ICSI (Ramasamy, et al., 2013). This study however did not report on LH and testosterone. Our findings support the hypothesis that deregulation of the hypothalamic–pituitary–gonadal axis may influence the quality of sperm in terms of the ability to conceive. Future research should explore whether or not paternal obesity and perhaps coinciding low testosterone levels effects live birth rates. Since in the majority of the cases the genetic cause of the azoospermia is still unknown (Ezeh, 2000), it is possible that genetic effects may also influence the live birth rate.

In our study sperm motility was found as an independent predictor for live birth. In ejaculated sperm, motility is a marker for sperm integrity and is associated with better chromatin condensation and less DNA-damage (Moskovtsev, et al., 2009, Ortega, et al., 2011, Ramos and Wetzels, 2001). It is unknown whether motility is also a marker for integrity of testicular sperm, since testicular sperm is not meant to be motile and chromatin condensation is incomplete.

The strength of our study is that we used data from the only two centres in the Netherlands who were allowed to perform TESE-ICSI until May 2014. Before the study period TESE-ICSI was not allowed due to restrictions in government policies based on concerns about the safety of the procedure for the men and health of the children born from the use of testicular sperm. It enabled us to develop and also externally validate a prediction model with live birth as primary outcome.

The applicability of our results in different clinical settings is subject to certain limitations. For instance, in this study we included all couples with azoospermic men, in which TESE was the only suitable method for sperm retrieval and who underwent TESE-ICSI, i.e. we did not differentiate between patients diagnosed with NOA and patients who were suspected of OA but with a previous failed PESA/MESA. We did not include a histologic diagnosis, which would have made our data set less heterogeneous. For those couples with an OA.NOA diagnosis (suspected OA but with no sperm retrieval after epididymal sperm aspiration), the sperm retrieval rate after TESE is higher. Our model may not be applicable in centres which have a different policy for the indication of performing PESA and TESE. Moreover, in these cycles the chance to find enough motile spermatozoa for injection of all oocytes, which we found as an independent predictive factor for live birth,

is also higher. Finally, the prognostic value of the model is limited because of a low 'area under the curve'.

In fertility treatment for couples with men diagnosed with azoospermia there are several steps to take, e.g. TESE, oocyte retrieval, successful thawing of TESE spermatozoa, injection of oocytes, fertilization, embryo transfer, pregnancy and finally giving birth. A clear starting point should be determined for developing a prediction model for these couples. In this study we choose this point after successful TESE; we included couples when the oocyte retrieval resulted in the injection of oocytes with testicular extracted spermatozoa. We have also developed a model which predicts the chance of finding spermatozoa in men with NOA (Cissen, et al., 2016). The predictive capacity our model was fair with an AUC of 0.69 in the development set and 0.65 in the validation set. The calibration indicated that our model could distinguish men with a poor prognosis from men with a good prognosis. Ideally, all parameters in a prognostic model are known before start of the treatment. However, we had a lack of data of the motility of spermatozoa at TESE. Therefore, we decided to use the motility of the spermatozoa used for ICSI. In the counselling of couples one should discuss both options, i.e. with or without motile spermatozoa used for ICSI. The motility of spermatozoa at TESE (before freeze thawing) may give an indication for the chance of having motile spermatozoa for ICSI. Sperm cells extracted after a first TESE, and remaining after a first use for TESE-ICSI, could be used in a second TESE-ICSI treatment. Our model provides some insight and may help in the counselling of couples about their chances of success during the fertility treatment and might help them in their decision whether or not to continue treatment after a failed cycle.

Without TESE-ICSI NOA couples do not have a chance to conceive. Physicians can involve patients in the process of making decisions about their health so that patients receive care that meets their needs and wishes. This is called 'shared decision-making' (Legare, et al., 2010). Using shared decisions, the physician and the couple can assess the burden of the treatment and the chance of a live birth (Baysal, et al., 2015). Several studies found that patients who are involved in decisionmaking are less prone to experience decisional conflict and regret (Bastings, et al., 2014, Legare, et al., 2010). Using our model a physician is better able to inform the couple about their actual chances.

In conclusion, in this study we developed and externally validated a prediction model which is able for the first time to predict the chance of live birth after TESE-ICSI. Using this model it is possible to counsel couples individually about their chances to conceive and becoming parents. Considering their individual chance to conceive and weighing their personal options will help couples in deciding to start or continue treatment or not. Our study revealed that besides the known factors, such as female age and the number of started cycles, a number of male-related factors (i.e. male LH, male testosterone, motility of spermatozoa used for ICSI and the initial male diagnosis) were also predictors for live birth after TESE-ICSI.

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4

Effect of maternal and treatment-related factors on the prevalence of birth defects after PESA-ICSI and TESE-ICSI: a retrospective cohort study

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Abstract

Introduction

We performed a retrospective cohort study with the aim to evaluate the effect of maternal and treatment-related factors on the prevalence of birth defects after intracytoplasmic sperm injection (ICSI) using percutaneous epididymal sperm aspiration (PESA) and testicular sperm extraction (TESE).

Material and methods

643 newborns born after PESA-ICSI (n = 406) and TESE-ICSI (n = 237) in Radboud University Medical Center, after a gestational age of 12 weeks, 1 January 2002–1 January 2011 and 1 March–1 November 2014, respectively, were included in this study. Three sources of data were used for analysis: questionnaires, national obstetrics registration forms, and a lab database of all ICSI treatments. Data were analyzed using generalized estimating equations and logistic regression analysis.

Results

The prevalence of major birth defects in newborns born after PESA-ICSI was 6.9% and after TESE-ICSI was 5.9% (odds ratio 0.89, 95% confidence interval 0.46–1.75). No significant association was found between maternal or treatment-related factors and the prevalence of birth defects.

Conclusions

We found a similar overall prevalence of birth defects in newborns born after PESA-ICSI and TESE-ICSI. The maternal and treatment-related factors investigated did not show a significantly increased cumulative risk of birth defects.

Introduction

Since 1992, intracytoplasmic sperm injection (ICSI) has become the preferred method of treatment in severe male infertility. In 10% of the male infertility cases, the male suffers from azoospermia. In these cases, it is only possible for couples to conceive by ICSI using either percutaneous epididymal sperm aspiration (PESA) for males suffering from obstructive azoospermia or testicular sperm extraction (TESE) for males suffering from non-obstructive azoospermia (Devroey, et al., 1994).

Generally, a slightly higher rate of birth defects is found in children born after assisted reproductive techniques (ART) than those conceived naturally (Pandey, et al., 2012). Recent studies regarding children born after PESA-ICSI or TESE-ICSI appear reassuring but they mainly focus on pregnancy and neonatal outcome (Fedder, et al., 2013, Tsai, et al., 2011, Woldringh, et al., 2010). However, not only infertility, and therefore ART, is associated with birth defects. Several studies show other influencing factors (de la Rochebrochard and Thonneau, 2002, Hackshaw, et al., 2011, Karacan, et al., 2013, Maheshwari, et al., 2012, Tsai, et al., 2013).

First, a higher risk of miscarriage and chromosomal defects in the offspring of women over 35 has been established (de la Rochebrochard and Thonneau, 2002). However, it remains unknown whether this risk of birth defects is more strongly influenced by maternal age after PESA-ICSI or TESE-ICSI.

Secondly, evidence confirms that congenital birth defects are associated with maternal smoking during pregnancy (Hackshaw, et al., 2011, Salmasi, et al., 2010).

A third factor may be the use of fresh or cryopreserved sperm. Studies investigated the differences between the use of fresh and cryopreserved non-ejaculated sperm on fertilization rates and perinatal outcome (Karacan, et al., 2013, Tsai, et al., 2013). None of these studies investigated the influence of using fresh or cryopreserved non-ejaculated sperm on birth defects.

Fourthly, the prevalence of birth defects might be influenced by embryo transfer (ET)-related factors such as the transfer of fresh or frozen-thawed embryos and the quality of the embryos. A meta-analysis showed a better perinatal outcome in singleton in vitro fertilization (IVF) and ICSI pregnancies after the transfer of frozen-thawed embryos, but these results were not specified for PESA or TESE groups (Maheshwari, et al., 2012).

There have been several studies on birth defects in children born after ICSI with non-ejaculated sperm; however, too little attention has been paid to other possible factors influencing the risk of birth defects. We expect that there may be worse outcomes for TESE children since immature sperm cells are used in TESE-ICSI. Therefore, we performed a cohort study with the following aims: to evaluate the prevalence of birth defects in newborns born after PESA-ICSI and TESE-ICSI, and to investigate the effect of maternal and treatment-related factors, i.e. maternal age, maternal smoking behaviour, fresh or frozen- thawed sperm, fresh or frozen-thawed embryos, and embryo quality.

Material and methods

Study design

A retrospective cohort study was performed. Newborns born between 1 January 2002 and 1 January 2011 (PESA-ICSI) and between 1 March 2008 and 1 November 2014 (TESE-ICSI) were included. During the study period in the Netherlands, PESA and TESE procedures were allowed only in research settings. Therefore, PESA-ICSI and TESE-ICSI study protocols were approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, The Hague, the Netherlands P99.000.1A & NL12408.000.06) and followed the Helsinki Declaration.

Study population

All newborns born after an ongoing pregnancy beyond 12 weeks' gestational age after PESA-ICSI (n = 421) or TESE-ICSI (n = 259) procedures in the Radboud University Medical Center were eligible for inclusion. All pregnancies occurred without interference of pre-implantation genetic diagnosis. In this study, both foetuses and children were analyzed collectively; we called this group 'newborns' unless stated otherwise. We subdivided foetuses born before and foetuses born after 24 weeks of gestational age because of viability after 24 weeks. All parents gave their written informed consent for treatment and follow up of newborns before participating in this study.

Before entering the fertility treatment procedure, each male underwent a complete andrologic evaluation by an urologist. Males with deletions in the AZF-a or AZF-b region of the Y-chromosome or chromosomal abnormalities were excluded for TESE. In the case of obstructive azoospermia, PESA was the primary choice of treatment. In the case of non-obstructive azoospermia or where no viable sperm was found after PESA, a TESE procedure was performed. Generally, in both obstructive and non-obstructive azoospermia patients, diagnostic sperm retrieval was performed in advance, to make sure that viable sperm were available. Retrieved sperm were cryopreserved. If sperm could not be cryopreserved in the diagnostic setting or no viable sperm were found after thawing, the procedure was repeated at the day of ovum pick up.

Women were admitted for an IVF/ICSI treatment at the fertility department. Exclusion and inclusion criteria were according to the local clinical protocol. Women were down regulated with a GnRH agonist following the standard long agonist protocol as described previously by Dam et al. (Dam, et al., 2012). The ovum pick up was performed 36 h after hCG injection when ≥three follicles with a diameter of >17 mm were observed using ultrasound examination. ICSI was performed as described previously by DeVroey et al. (Devroey, et al., 1994). Transfer policy has changed over time, in the last 3 years towards compulsory single ET in women <38 years. Frozen embryos were mainly transferred in hormonally substituted cycles. In the case of pregnancy, presence of foetal cardiac activity was examined at a gestational age of 7–8 weeks.

Data collection

Three sources of data were used for the follow up of all newborns. First, data was obtained from the ART treatment lab-database at the Radboud University Medical Center with regard to embryos (fresh or frozen-thawed, number of embryos transferred, embryo quality) and sperm (PESA/TESE, fresh or frozen-thawed). Secondly, parents were asked to fill in questionnaires after birth and after 1 year. These questionnaires collected parental data (maternal age, maternal and paternal smoking behaviour), on pregnancy (such as hypertension, preeclampsia, gestational diabetes), labour (such as complications, position, assisted delivery) and child-related data (such as birth defects). Questions regarding smoking behaviour were specified by asking whether the mother smoked before or during pregnancy, and whether the father smoked at all (passive smoking). Only mothers who smoked during pregnancy were assigned to the smoking group. If parents did not respond to the questionnaire, they received a reminder 3 months later. A third additional data source was collected via an obstetric registration form provided by hospitals and midwifes after delivery to collect extra medical information. This form gathered information about medical problems in pregnancy, APGAR scores and reported information about the presence of birth defects. However, no additional birth defects were mentioned in the obstetric registration form.

If a questionnaire was not returned, the children were considered lost to follow up and were excluded from analysis.

Definitions

Embryo scoring and evaluation was based on fragmentation and the number of blastomeres using a light inverted microscope determined by a qualified embryologist or lab technician on day 3. The quality of each embryo was categorized from best to lowest as follows: A-quality: embryos with 0–20% fragmentation and containing 7, 8 or 9 blastomeres; B-quality: embryos with 20–50% fragmentation containing 7 or 8 blastomeres, or embryos with 0–20% fragmentation containing 4, 5, 6 or 10, 11, 12 blastomeres; C-quality: all remaining embryos. After scoring, one or two of the best embryo(s) available were selected for ET. In the case of double ET, only the best available embryo quality was taken into account in our analyses. Birth defects were defined as all congenital abnormalities that are present in less than 4% of the general population of the same racial group (Smith, 1975).

Birth defects were classified according to the International Classification of Disease (ICD-10) and were also classified according to the severity of major and minor birth defects (Bonduelle, et al., 2002).

Major birth defects were defined as those birth defects that generally cause functional impairment or require surgical correction, with the following exceptions: pyloric stenosis was considered major; inguinal hernia was considered major for a child born at term (≥37 weeks) but minor for a child born preterm; ductus arteriosus was considered major if still

present after 3 months for a child born at term or still present after 6 months for a child born preterm. The remaining birth defects were considered to be minor (Smith, 1975).
Infant death was subdivided into three groups: early neonatal death for children who died within 7 days; late neonatal death for children who died within 7–28 days; post neonatal death for children who died between 28 days and 1 year (Barfield, et al., 2011).

Statistics

First, birth characteristics were described, separately for singletons and multiples. Continuous data were compared using Student's t-test if the distribution was considered sufficiently normal or Mann–Whitney U-test if not. Fisher's exact test was used for nominal outcome data. The association between the presence of major birth defects and maternal and treatment-related factors was evaluated by means of a generalized estimating equations approach for binomial data using a logit link, resulting in odds ratios (OR) and 95% confidence intervals (CI). Two-sided p-values < 0.05 were considered significant. To correct the models for a possible correlation in the outcomes due to the fact that a mother could have multiple children, an exchangeable working correlation matrix was used. If there were no birth defects in one of the groups, a continuity correction was applied by allocating 0.1 to one of the subjects of that group in order to be able to estimate an OR. All factors were analyzed separately for the PESA and TESE groups. To evaluate the effect of the combined factors, we performed first an univariate analysis in the total groups of newborns followed by an explorative multivariable analysis starting with all factors and PESA/TESE. Backward selection was applied manually. At all times, PESA/TESE, the main factor for analysis, was kept in the selection. With the quasi-likelihood under the independence model criterion it was decided whether maternal age should be added as a continuous or a dichotomized variable. Statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA).

Results

In total, 680 newborns were born after PESA-ICSI and TESE-ICSI, at a gestational age of at least 12 weeks, between 1 January 2002 and 1 January 2011 and between 1 March and 1 November 2014, respectively. Of these, 37 children were lost to follow up. As result, 643 newborns, PESA (n = 406) and TESE (n = 237), from 546 pregnancies of 465 mothers were included for analysis in this study. In all, 161 newborns were born from 80 mothers with two or more consecutive pregnancies.
Figure 1 shows a flow chart with the included newborns. In the TESE group, the group of multiples includes only twins, with the exception of one triplet. In the PESA group, three pregnancies were terminated, two because of trisomy 21 (maternal ages 32 and 39 years) and one because of trisomy 18. One pregnancy was terminated because of anencephaly

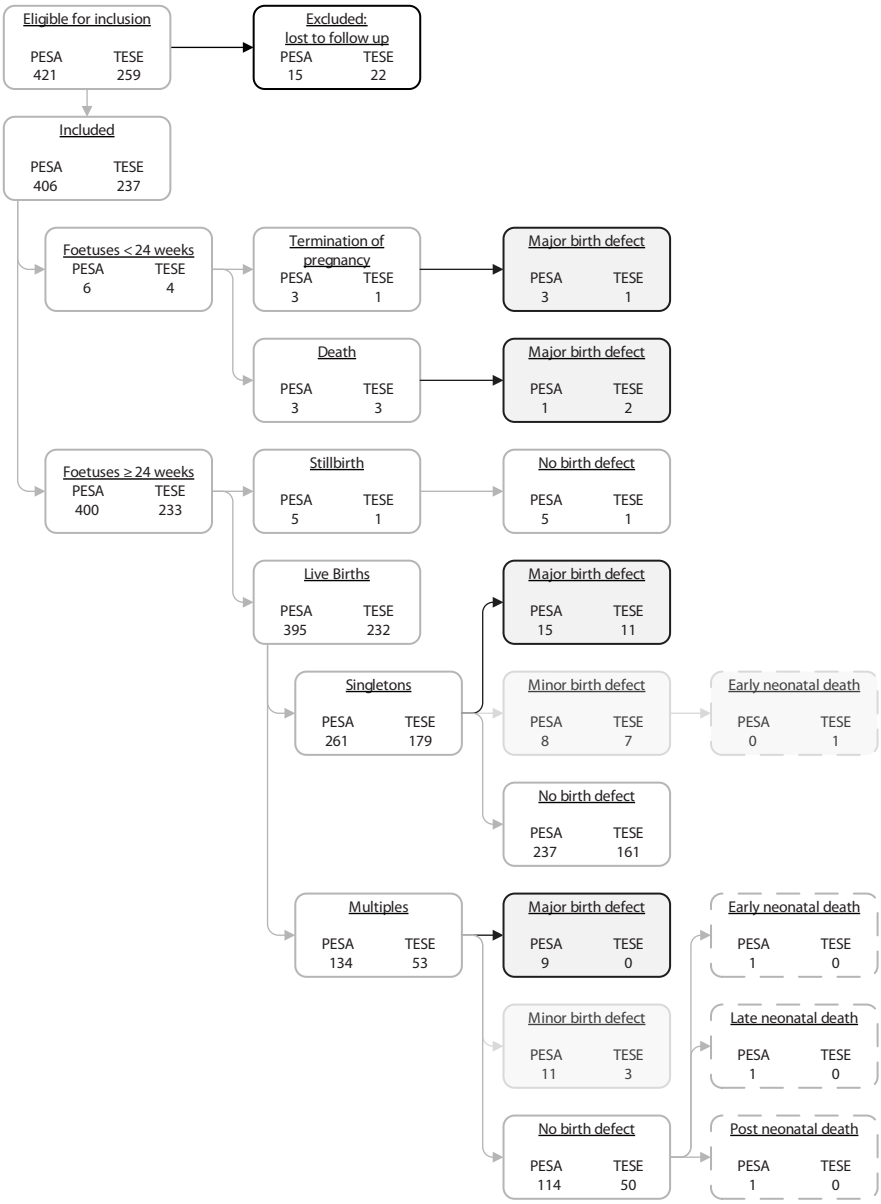

Figure 1 Flowchart of included newborns

in the TESE group. One twin pregnancy in the PESA group was reduced to a singleton pregnancy because of a child with a partial trisomy 9. In the group of foetuses <24 weeks' gestational age there were two miscarriages (<20 weeks). In both, a major birth defect was observed: a trisomy 18 (TESE) and the presence of an omphalocele (TESE). In the PESA group, one foetus was live born but died after an hour and a half. Infant death occurred in four cases. No major birth defects were found in these children. In two cases, the children suffered from necrotizing enterocolitis. All of these children were born preterm – one extremely preterm and three very preterm.

Table 1 shows the birth characteristics of all newborns. The prevalence of newborns with a major birth defect was 6.9% (28/406) in the PESA group and 5.9% (14/237) in the TESE group. The effect of the ART procedure was not significant (OR TESE vs. PESA 0.89, CI 0.46–1.75). No significant differences were found in birth weight, gender, APGAR score or gestational age between the PESA and TESE group for singletons and multiples, respectively.

Table 2 presents an overview of major and minor birth defects specified per tract for both treatments. If a newborn had multiple birth defects, the defects are mentioned separately to provide insight into the prevalence of birth defects in each tract. In the PESA group, one child was born at term and was diagnosed with an inguinal hernia (K40.3); this child also suffered from microcephaly, dysmorphic features and psychomotor retardation e causa ignota (F79.0). Another child in this group was born preterm and had an inguinal hernia (K40.9) and also was diagnosed with macrocephaly-capillary malformation syndrome (Q87.3). The TESE group also contained a child with two major birth defects. In this child, an anus atresia (Q42.2) and a bicuspid aortic valve (Q23.8) were observed. The musculoskeletal tract was the most affected tract in both groups, with hip dysplasia (Q65.x) providing the largest share: PESA, n = 5 (17%) and TESE, n = 5 (33%). In each group, two children with hip dysplasia had a breech position during delivery.

Table 3 presents the ORs for the association of various maternal and treatment-related factors in relation to birth defects in the separate PESA and TESE groups and in the whole group. There was no significant effect of maternal age. Maternal smoking behaviour, such as maternal smoking during pregnancy, did not notably influence the prevalence of birth defects. Of the non-smoking group, 105 mothers had a partner who smoked, of which four newborns (3.8%) suffered from a major birth defect. Sperm- and embryo-related factors did not show a significant influence on the prevalence of birth defects.

After analyzing all factors separately in the PESA/TESE groups and in the whole group, an explorative multivariable model was built starting with all factors, PESA/TESE, and maternal age as continuous variables. Using backward selection, all factors were eliminated from the model except the PESA/TESE group, which was kept on purpose. None of the factors had a significant association with birth defects in PESA and TESE groups.

Table 1 Characteristics of the newborns

	PESA			TESE			PESA & TESE
	Singletons	**Multiples**	**Total**	**Singletons**	**Multiples**	**Total**	**Total**
Total newborns	**266**	**140**	**406**	**184**	**53**	**237**	**643**
Major birth defects n (%)	18 (6.8)	10 (7.1)	28 (6.9)	14 (7.6)	0	14 (5.9)	42 (6.5)
Live borns	**261**	**134**	**395**	**179**	**53**	**232**	**627**
Gender							
Boys n (%)	128 (49.0)	59 (44.0)	187 (47.3)	92 (51.4)	26 (49.1)	118 (51.1)	305 (48.6)
Girls n (%)	133 (51.0)	75 (56.0)	208 (52.7)	87 (48.6)	27 (50.9)	114 (48.9)	322 (51.4)
Birth weight							
Mean in grams (SD)	3423 (657)	2362 (692)	3063 (836)	3419 (560)	2331 (579)	3170 (726)	3103 (798)
APGAR score							
Mean after 5 min (SD)	9.6 (0.7)	9.2 (1.2)	9.5 (1.0)	9.7 (0.8)	9.2 (1.0)	9.6 (0.9)	9.5 (0.9)
Unknown n (%)	69 (26.4)	22 (16.4)	91 (23.0)	65 (36.3)	15 (28.3)	80 (34.6)	171 (27.3)
Gestational age[a]							
Mean in weeks (SD)	39.4 (2.2)	35.8 (3.2)	38.2 (3.1)	39.5 (2.0)	36.2 (2.8)	38.7 (2.6)	38.4 (2.9)
<28 weeks n (%)	1 (0.4)	0	1 (0.3)	1 (0.6)	0	1 (0.4)	2 (0.3)
28+0–31+6 weeks n (%)	2 (0.8)	18 (13.4)	20 (5.1)	1 (0.6)	7 (13.2)	8 (3.5)	28 (4.5)
32+0–36+6 weeks n (%)	18 (6.9)	49 (36.6)	67 (17.0)	8 (4.5)	16 (30.2)	24 (10.4)	91 (14.5)
37+0–41+6 weeks n (%)	228 (87.4)	66 (49.3)	294 (74.4)	166 (92.7)	30 (56.6)	196 (84.8)	490 (78.1)
≥42 weeks n (%)	12 (4.6)	1 (0.7)	13 (3.3)	3 (1.7)	0	3 (1.3)	16 (2.6)

[a]Gestational age is mentioned for each live born newborn separately.

Table 2 Major birth defects specified per tract and treatment

	PESA		TESE	
	Total n (%)	**Major n (%)**	**Total n (%)**	**Major n (%)**
Total number of newborns	47	28	24	14
Circulatory tract	6 (13)	3 (11)	3 (13)	2 (14)
PESA: Q21.3;Q25.1				
TESE: Q21.0; Q23.8				
Respiratory tract	3 (6)	2 (7)	0	0
PESA: Q32.1				
Gastrointestinal tract	9 (19)	2 (7)	1 (4)	1 (7)
PESA: K40.3; Q40.0				
TESE: Q42.2				
Urogenital tract	4 (9)	1 (4)	4 (17)	1 (7)
PESA: Q60.0				
TESE: Q60.4				
Musculoskeletal tract	11 (23)	9 (32)	8 (33)	7 (50)
PESA: Q65.2; Q65.5; Q66.0; Q70.4				
TESE: Q65.2; Q65.6; Q66.0; Q79.2				
Nervous system	2 (4)	1 (4)	1 (4)	1 (7)
PESA: Q03.9				
TESE: Q00.0				
Skin	4 (9)	1 (4)	5 (21)	2 (14)
PESA: Q38.6				
TESE: D22.9; Q82.0;				
Chromosomal	4 (9)	4 (14)	1 (4)	1 (7)
PESA: Q90.9; Q91.3; Q92.9				
TESE: Q91.3				
Other	5 (11)	5 (18)	2 (8)	0
PESA: E03.1; E71.3; E88.9; Q77.3; Q87.3				

ICD-10: Major birth defects were classified according to ICD-10, codes are corresponding with disease (appendix). If a newborn had multiple birth defects, the defects are mentioned separat*ely*

Table 2a Minor birth defects specified per tract and treatment

	PESA		TESE	
	Total n (%)	**Minor n (%)**	**Total n (%)**	**Minor n (%)**
Total number of newborns	47	19	24	10
Circulatory tract	6 (13)	3 (16)	3 (13)	1 (10)
PESA: Q21.0; Q25.0				
TESE: Q25.0				
Respiratory tract	3 (6)	1 (5)	0	0
PESA: Q30.0				
Gastrointestinal tract	9 (19)	7 (37)	1 (4)	0
PESA: K40.2; K40.9; K42.9				
Urogenital tract	4 (9)	3 (16)	4 (17)	3 (30)
PESA: P83.5; Q62.5; Q63.9				
TESE: Q52.4; Q53.1; Q53.2				
Musculoskeletal tract	11 (23)	2 (11)	8 (33)	1 (10)
PESA: Q66.0; Q69.0				
TESE: Q69.0				
Nervous system	2 (4)	1 (5)	1 (4)	0
PESA: Q13.1				
Skin	4 (9)	3 (16)	5 (21)	3 (30)
PESA: Q82.5				
TESE: Q82.5; Q82.9				
Chromosomal	4 (9)	0	1 (4)	0
Other	5 (11)	0	2 (8)	2 (20)
TESE: Q17.0				

ICD-10: Minor birth defects were classified according to ICD-10, codes are corresponding with disease (appendix)
If a newborn had multiple birth defects, the defects are mentioned separat*ely.*

Table 3 Major birth defects in relation to maternal and treatment related factors

	PESA				TESE				PESA&TESE	
	No birth defect n (%)	**Birth defect n (%)**	**OR**	**95%CI**	**No birth defect n (%)**	**Birth defect n (%)**	**OR**	**95%CI**	**OR**	**95%CI**
Total newborns	378 (93.1)	28 (6.9)	-	-	223 (94.1)	14 (5.9)	-	-	0.89	0.46-1.75
Maternal factors										
Maternal age										
<35 years	252 (92.6)	20 (7.4)	1.00	-	161 (94.2)	10 (5.8)	1.00	-	1.00	-
≥35 years	126 (94.0)	8 (6.0)	0.83	0.35-1.96	62 (93.9)	4 (6.1)	1.03	0.31-3.41	0.89	0.44-1.79
Maternal smoking [a]										
No smoking	357 (93.2)	26 (6.8)	1.00	-	215 (93.9)	14 (6.1)	1.00	-	1.00	-
Smoking	21 (91.3)	2 (8.7)	1.30	0.30-5.57	8 (100)	0	0.18	0.02-1.39	0.95	0.23-3.93
Sperm related factors										
Sperm										
Fresh	189 (94.0)	12 (6.0)	1.00	-	38 (92.7)	3 (7.3)	1.00	-	1.00	-
Cryo	189 (92.2)	16 (7.8)	1.36	0.62-3.00	185 (94.4)	11 (5.6)	0.76	0.20-2.87	1.18	0.58-2.41
Embryo related factors										
Embryo										
Fresh	347 (93.3)	25 (6.7)	1.00	-	215 (93.9)	14 (6.1)	1.00	-	1.00	-
Cryo	31 (91.2)	3 (8.8)	1.23	0.30-5.12	8 (100)	0	0.19	0.03-1.31	1.00	0.26-3.81
Embryo quality										
A-quality	235 (94.4)	14 (5.6)	1.00	-	148 (93.7)	10 (6.3)	1.00	-	1.00	-
B-quality	134 (90.5)	14 (9.5)	1.83	0.87-3.88	63 (95.5)	3 (4.5)	0.70	0.19-2.63	1.38	0.74-2.57
C-quality	9 (100)	0	0.23	0.04-1.27	12 (92.3)	1 (7.7)	1.21	0.14-10.40	0.75	0.10-5.42

Presented odds ratios (OR) and confidence intervals (CI) are corrected for the inclusion of multiple children (and foetuses) conceived by one mother, presented numbers and percentages are uncorrected .a Maternal smoking by the mother during pregnancy.

Discussion

The aim of this study was to evaluate the effect of maternal and treatment-related factors on the prevalence of birth defects after PESA-ICSI and TESE-ICSI. We found no significant difference in the prevalence of major birth defects between the TESE group (5.9%) and the PESA group (6.9%). None of the factors – maternal age, maternal smoking behaviour, fresh or frozen-thawed sperm, fresh or frozen-thawed embryos and embryo quality – had a significant effect on the prevalence of birth defects.

The main result of this study, regarding the similar prevalence of birth defects between PESA-ICSI and TESE-ICSI, is consistent with previous studies (Belva, et al., 2011, Woldringh, et al., 2010). Others found a prevalence of 6.2% major birth defects in 1462 children born after IVF and 4.4% in 8422 children born after spontaneous conception (Olson, et al., 2005). The prevalence of birth defects in our study is in line with that in the IVF children, and slightly higher than in children born after spontaneous conception.

In ART using non-ejaculated sperm, some steps have to be taken before an ET can take place. These steps, i.e. PESA or TESE, cryopreserving sperm, ICSI, embryo quality selection and cryopreserving embryos, separately are found to be related to an increase in prevalence of birth defects (Karacan, et al., 2013, Maheshwari, et al., 2012, Pandey, et al., 2012). In addition, external factors, such as maternal age and maternal smoking behaviour, are often investigated in relation to the risk of birth defects (de la Rochebrochard and Thonneau, 2002, Hackshaw, et al., 2011). However, little is known about the cumulative effect of these maternal and treatment-related factors in combination with this multiplicity of procedures.

First, advanced maternal age is recognized as a risk factor for birth defects above the age of 40 (Vaughan, et al., 2014). In our analyses, the group of women above 40 years was small due to the maternal age-related exclusion criterion for ICSI. Therefore, the cut-off point for advanced maternal age was set at 35 years based on the literature, which states that the risk of both chromosomal and non-chromosomal birth defect rises substantially with advanced maternal age, i.e. >35 years (Reefhuis and Honein, 2004, Yoon, et al., 1996). We did not observe an increase in birth defects in this group.

Another known factor of influence is maternal smoking behaviour. A recent systematic review showed a significant increase in birth defects in the musculoskeletal tract, the gastrointestinal tract and birth defects involving the eyes and the oral cleft, in children of mothers who smoked during pregnancy (Hackshaw, et al., 2011). This finding could not be reproduced in our study. However, maternal smoking during pregnancy was reported by only 4.8% of women in our study population. This is substantially lower than the Dutch average of 7% (van Gelder, et al., 2008) and the average of 17% in the systematic review (Hackshaw, et al., 2011). This could be due to the wish to conceive and preconception or prenatal counselling women received. However, bias due to data collection by self-reported questionnaires cannot be ruled out. Salmasi et al. found an increased risk of

birth defects in children born from passive smoking mothers (Salmasi, et al., 2010). Nevertheless, in the passive smoking group in our study, the prevalence of birth defects was only 3.8%.

The freeze-thawing process is known to have a major impact on viability of sperm. However, this effect seems to apply mostly to the quantity of sperm cells. There is no indication of a negative effect on the quality of the sperm cells surviving the freeze-thawing process (Tsai, et al., 2013, Wald, et al., 2006). Previous research did not find negative effects on fertilization rates or pregnancy rates when using frozen-thawed sperm (Friedler, et al., 1997, Habermann, et al., 2000). We found no significant increase of birth defects observed after using cryopreserved sperm. We hypothesize that these reassuring findings might be the consequence of the semi-natural selection through the freeze-thawing procedure, leaving a lower quantity of sperm cells capable of generating similar results as a full number of fresh sperm (Karacan, et al., 2013, Tsai, et al., 2013). This could imply that the highest quality sperm cells survive the freeze-thawing process.

We found no difference in birth defects between newborns conceived with fresh and frozen-thawed embryos. A meta-analysis found the same results in the IVF population (Maheshwari, et al., 2012). The reason for similar or even better outcomes of frozen-thawed embryos than fresh embryos remains unknown. Better outcomes were judged mainly on birth weight, gestation and the occurrence of antepartum haemorrhage and mortality in IVF/ICSI studies (Maheshwari, et al., 2012). As we suggested for freeze-thawing of sperm, the physical effects of the freeze-thawing procedure may be a way of selecting embryos of the best quality (Maheshwari, et al., 2012). In addition, there is no ovarian hyperstimulation needed immediately prior to an ET with a frozen-thawed embryo. Therefore, the better outcome after cryo ET might suggest that the more natural endometrial angiogenesis favours the implantation and development of embryos.

The last factor of interest was embryo quality. Fauque et al. showed a significant influence of embryo quality on pregnancy and live birth rates in IVF and ICSI (Fauque, et al., 2007), but does not discuss the influence of embryo quality on the prevalence of birth defects. Our study showed no significant effect on the occurrence of birth defects. The majority of newborns were born after an ET with an A or B-quality embryo; only 3.4% of the newborns included were born after an ET of a C-quality embryo. Although not significant, a slightly higher prevalence of birth defects was found in the B-quality group than in the A-quality group.

The distribution of birth defects over various tracts was comparable to that in previous studies (Bonduelle, et al., 2002, Fedder, et al., 2013). We found most birth defects originated from the musculoskeletal tract. In particular, hip dysplasia was the largest contributor within the group of newborns with a birth defect [PESA, n = 5 (17%) and TESE, n = 5 (33%)]. Although this was the largest share of all birth defects, the prevalence (PESA 1.2% and TESE 2.1%) seems similar to the prevalence in spontaneous conceived children (1.4–3.5%) (Lambeek, et al., 2013, Lehmann, et al., 2000, Witt, 2003). Regarding the distribution of birth

defects, an increased prevalence of hypospadias, as found by Fedder et al., could not be confirmed (Fedder, et al., 2007). However, in that study only three cases of hypospadia were found, of which two were from dizygotic twins.

Our study has some limitations. A major limitation is the rather low power of the study due to a low prevalence of birth defects. We performed a post-hoc power calculation, based on the actual group sizes of PESA and TESE and the actual birth defect rate of 6.9% in the PESA group. This showed that a difference of approximately 7% in birth defect prevalence could have been detected with 80% power and a significance level of 5%.

Another limitation is that data were collected by parent reported questionnaires. This questionnaire was used in another study (Woldringh, et al., 2011). In that study, the same questionnaires were sent after birth and after 1 year, and physical examination was performed at 2 years. The authors found that after 1 year, parents reported additional birth defects. However, at 2 years of age, physical examination revealed no birth defects other than those already reported in the questionnaires. Therefore, it seems that the parents reliably reported birth defects correctly. Furthermore, the conclusions involving the embryo quality may have been influenced by cases with double ET in which two embryos with different embryo quality were used. Newborns could not be linked to the original embryo with 100% certainty.

The strength of this study lies in the included maternal and treatment-related factors. We studied the influence of each factor separately, with the consideration of other possible confounding factors. Based on our results, our study rejects the hypothesis that TESE-ICSI increases the prevalence of major birth defects, in comparison with PESA-ICSI, in terms of maternal and treatment-related factors. However, the number of newborns included was small to draw statistically significant conclusions about the influence of these factors. In conclusion, we found a similar overall prevalence of birth defects in newborns born after PESA-ICSI and TESE-ICSI. Additionally, the maternal and treatment-related factors investigated did not show a significantly increased cumulative risk of birth defects.

Appendix ICD-10 codes

Circulatory tract	
Q21.0	Ventricular septal defect
Q23.1	Congenital insufficiency of aortic valve
Q23.8	Other congenital malformations of aortic and mitral valves
Q25.0	Patent ductus arteriosus
Q25.1	Coarctation of aorta
Respiratory tract	
Q30.0	Choanal atresia
Q32.1	Other congenital malformations of trachea
Gastointestinal tract	
Q40.0	Congenital hypertrophic pyloric stenosis
Q42.2	Congenital absence, atresia and stenosis of anus with fistula
K40.2	Bilateral inguinal hernia, without obstruction or gangrene
K40.3	Unilateral or unspecified inguinal hernia, with obstruction, without gangrene
K40.9	Unilateral or unspecified inguinal hernia, without obstruction or gangrene
K42.9	Umbilical hernia without obstruction or gangrene
Urogenital tract	
P83.5	Congenital hydrocele
Q52.4	Other congenital malformations of vagina
Q53.1	Undescended testicle, unilateral
Q53.2	Undescended testicle, bilateral
Q60.0	Renal agenesis, unilateral
Q60.4	Renal hypoplasia, bilateral
Q62.5	Duplication of ureter
Q63.9	Congenital malformation of kidney, unspecified
Musculoskeletal tract	
Q65.2	Congenital dislocation of hip, unspecified
Q65.5	Congenital subluxation of hip, unspecified
Q65.6	Unstable hip
Q66.0	Talipes equinovarus
Q69.0	Accessory finger(s)
Q70.4	Polysyndactyly
Q79.2	Reduction defect of lower limb, unspecified
Nervous system	
Q00.0	Anencephaly
Q03.9	Congenital hydrocephalus, unspecified
Q13.1	Absence of iris

Appendix Continued

Skin	
D22.9	Melanocytic naevi, unspecified
Q38.6	Other congenital malformations of mouth
Q82.0	Hereditary lymphoedema
Q82.5	Congenital non-neoplastic naevus
Q82.9	Congenital malformation of skin, unspecified
Chromosomal	
Q90.9	Down syndrome, unspecified
Q91.3	Edwards syndrome, unspecified
Q92.9	Trisomy and partial trisomy of autosomes, unspecified
Other	
E03.1	Congenital hypothyroidism without goitre
E71.3	Disorders of fatty-acid metabolism
E88.9	Metabolic disorder, unspecified
F79.0	Unspecified intellectual disabilities
Q17.0	Accessory auricle
Q77.3	Chondrodysplasia punctata
Q87.3	Congenital malformation syndromes involving early overgrowth

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5

Behavioural, cognitive and motor performance and physical development of five-year-old children born after intracytoplasmic sperm injection with testicular sperm

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Abstract

Objective: To evaluate behavioural, cognitive and motor performance, and physical development of children born after TESE-ICSI at the age of five.
Design: A prospective longitudinal cohort study.
Setting: Two university medical centres.
Patients: A total of 103 five-year-olds born after TESE-ICSI.
Intervention(s): The follow-up of the children was performed by questionnaires at birth and again at one year old and at four years old. Five-year-old children were invited for individual assessment. Behavioural performance was assessed using the Child Behaviour Checklist for parents and teachers. Cognitive performance was assessed using the Dutch Wechsler Preschool and Primary Scale of Intelligence test, third version. Motor performance was assessed using the Dutch Movement Assessment Battery for Children, second version. Physical development was assessed by physical examination and medical history.
Main Outcome Measure(s): Behavioural, cognitive and motor performance, and physical development.
Results: Eighty-nine children were completely assessed and fourteen were partially assessed at the age of five. Our 'five-years-old cohort' assessed significantly better on behavioural and cognitive performance and significantly worse on motor performance—but still in the normal range—compared to the theoretical distribution in the general population. Four children (3.8%) of the 'five-years-old cohort' had developmental problems/delays. Two of them were previously diagnosed with a form of autism (Pervasive Developmental Disorder-Not Otherwise Specified). Two children had developmental problems based on our behavioural, cognitive and/or motor assessments.
Conclusion: The long-term effects on development and health in children born after TESE-ICSI procedures seem reassuring.

Introduction

For severe male factor infertility, intracytoplasmic sperm injection (ICSI) has become the method of treatment. Azoospermia, the most severe form of spermatogenesis abnormality, is found in about ten percent of all male infertility cases (De Croo, et al., 2000). When testicular sperm extraction (TESE) is successful, it is possible for those couples to conceive after TESE-ICSI. Since 1995 that method has been used worldwide. However, the presumed abnormal maturation of sperm retrieved from the testicles may have consequences for the development or health of the offspring born after TESE-ICSI.

A number of studies described pregnancy and neonatal outcomes of children born after TESE-ICSI shortly after birth. Those descriptions used data analysis (Fedder, et al., 2013, Oldereid, et al., 2014, Vernaeve, et al., 2003) as well as physical examination (Ludwig and Katalinic, 2003) by reporting on congenital abnormalities (Belva, et al., 2011, Fedder, et al., 2007). Fedder et al. reported an increased frequency of hypospadia but overall these studies were reassuring (Fedder, et al., 2007).

Long-term effects on development and health of children born from TESE-ICSI is a subject less studied. Many diseases or developmental problems manifest themselves later and are not visible or measurable at birth or shortly thereafter. Only two studies have focused on development of children during childhood (Bonduelle, et al., 2002, Tsai, et al., 2011). Bonduelle et al. examined physical, neurological and psychomotor development in a group of 206 children born after TESE-ICSI until the age of two, but the researchers did not report that data, focusing instead on congenital abnormalities (Bonduelle, et al., 2002). Tsai et al. used a preschool developmental screening table in a group of 60 children but mentioned only that no psychomotor or intellectual development retardations were noted. Moreover, the researchers used a retrospective cross-sectional design (Tsai, et al., 2011) and did not report on psychomotor and intellectual performance. A Dutch national cohort study regarding the development of very preterm of very low birth weight infants until the age of 19, reported that problems with behaviour and school performance were often noticed in the first years at school (van der Pal-de Bruin, et al., 2015).

Early childhood is a period of major neurocognitive development, Mous et al. found that intelligence quotient is positively associated with neuropsychological functioning in children aged 6 to 10 years (Mous, et al., 2016). Another study found that in term-born children, a low stability of motor development from birth until school age (Roze, et al., 2010). A Dutch national cohort study including 1338 very preterm of very low birth weight infants with a follow up until the age of 19, reported that problems with behavior and school performance were often noticed in the first years at school. Special education after the age of five was associated with mild problems with cognition, behavior and learning (van der Pal-de Bruin, et al., 2015). To our knowledge no prospective studies have been performed regarding behavioural, cognitive and motor performance, and physical development of children born after TESE-ICSI above two years of age.

Our aim of this study was to evaluate behavioural, cognitive and motor performance and physical development of children born after TESE-ICSI at the age of five. To that end we performed a nationwide, prospective longitudinal cohort study in the Netherlands during an eight year period.

Material and methods

Study design

A prospective longitudinal cohort study was performed on the development of children born after TESE-ICSI in the Radboud university medical center (Radboudumc) and the Academic Medical Center (AMC) between March 1, 2008, and May 1, 2016.

Ethical approval

The Dutch Central Committee on Research Involving Human Subjects (NL12408.000.06 CCMO, The Hague, the Netherlands) approved the protocol for this multicentre study.

TESE

Each male with azoospermia underwent a complete workup by an andrology-specialized urologist. In case of non obstructive azoospermia (NOA) or no viable spermatozoa were found after percutaneous epididymal sperm aspiration (PESA) or microsurgical epididymal sperm aspiration (MESA), a TESE procedure was performed, and the testicular sperm obtained was cryopreserved for further use with ICSI (Meijerink, et al., 2016).

TESE-ICSI

Each female partner was assessed for suitability for treatment before undergoing ICSI. Female patients were, in general, downregulated with a gonadotropin-releasing hormone agonist following the standard long agonist protocol as described previously by Dam et al. (Dam, et al., 2012). ICSI was performed as previously described by Palermo et al. (Palermo, et al., 1992). Three days after ovum pick up one or two embryos were transferred. The remaining high-quality embryos were frozen with a slow controlled-rate freezing procedure. Frozen embryos were mainly transferred in E2/progesterone substituted cycles. The presence of foetal cardiac activity was examined by ultrasound at a gestational age of seven to eight weeks.

Study procedures

All live born children after TESE-ICSI procedures in Radboudumc between March 1, 2008, and May 1, 2016, and live born children after TESE-ICSI procedures in AMC between March 1, 2008, and October 1, 2012, were eligible for inclusion. Participants signed informed consent forms before the TESE procedure was started. The follow-up of the children born

after TESE-ICSI treatment was performed by questionnaires at birth and at one year and four years of age. The questionnaire after birth collected data on parental (age, smoking behaviour, educational level), pregnancy (e.g., hypertension, preeclampsia, gestational diabetes), and labour and child factors (including gestational age, mode of delivery, birth weight, presence or absence of malformations, and neonatal problems). The questionnaire at one and four years of age collected data regarding growth parameters and medical and paramedical history. If parents did not respond to the questionnaire they received a reminder letter three months later.

Assessment of the 'five-years-old cohort' children

In addition to the questionnaires, the five-year-old children were invited for complete behavioural, cognitive, motor and physical assessments. In the general population these assessments are only performed when indicated, in this study we used them to evaluate development in our 'five-years-old cohort'. The children's assessments took place in a single visit and were administered by a paediatric psychologist, a paediatric physiotherapist and a physician in Radboudumc. If the parents refused to come to the hospital for the assessments, they were asked if they would agree to partial assessments by completing questionnaires about medical condition and behaviour or by providing information from a general practitioner or paediatrician. Together, this complete and partially assessed group we named 'five-years-old cohort'.

Assessment of behavioural performance

Behavioural performance was assessed by using the Dutch version of the Child Behaviour Checklist (CBCL) for children aged 1.5–5 years as reported by both parents, and the Teacher Report Form (TRF) as reported by the teacher (Achenbach, 2000). The CBCL and the TRF are validated questionnaires and both rate problem behaviour items on a 3-point scale based on the behaviour of the child during the preceding 2 months. Scores of the questionnaires were recalculated into age-corrected T-scores. The T-scores of our study cohort were compared with the theoretical distribution of the CBCL en TRF based on a normative value of 50 and a standard deviation of 10. T-scores were categorised from best to worst as follows: normal range, <60; borderline range, 60–63; and clinical range, ≥63. The scales of the CBCL and the TRF distinguish internalizing and externalizing problems (Piek, et al., 2010). The total test score includes all problem scales.

Assessment of cognitive performance

Cognitive performance was assessed by using the Wechsler Preschool and Primary Scale of Intelligence-III-NL test (WPPSI-III-NL) for children aged 4.0–7.1 years old and was administered by a paediatric psychologist. The WPPSI-III-NL is a validated test which distinguishes total intelligence quotient (IQ), verbal IQ, performal IQ and processing speed (Hurks, et al., 2013). The age-corrected scores of our study cohort were compared with the

theoretical distribution of the WPPSI-III-NL test scores based on a normative value of 100 and a standard deviation of 15.

Assessment of motor performance

Motor performance was assessed using the Movement Assessment Battery for Children, second Dutch version (MABC-2-NL) (Henderson, et al., 2010). This test assesses manual dexterity, aiming and catching and balance motor tasks. The scores of those three components were converted to a standard total test score. Children with a standard total test score of >7 were considered to have a normal motor performance. Children with a score of 6 or 7 were considered to be at risk of having a movement difficulty. Children with a score of ≤5 were considered to be at risk of a significant movement difficulty. The age-corrected standard scores of our study cohort were compared with the theoretical distribution of the MABC-2 NL standard scores based on a normative value of 10 and a standard deviation of 3.

Assessment of physical development

Physical development was assessed by medical physical examination (i.e., inspection of birth defects, inspection of the skin, auscultation, basic neurologic examination, length and weight) and asking parents questions about the medical history of their child during the past year. Standard deviations for length, weight and weight for length were calculated with the growth curves of boys and girls of the same age and racial group.

Definitions

A birth defect was defined as a certain congenital abnormality that is present in less than four percent of the general population of the same racial group (Holmes, 1976, Smith, 1975). Birth defects were classified according to the International Classification of Disease (ICD-10). Major birth defects were defined as those birth defects that generally cause functional impairment or require surgical correction, with the following exceptions: Pyloric stenosis was considered major; inguinal hernia was considered major for a child born at term but minor for a child born preterm; and ductus arteriosus was considered major if it was still present after three months for a child born at term or still present after six months for a child born preterm. The remaining birth defects were considered minor (Bonduelle, et al., 2002).

Gestational age was subdivided into groups following the accepted definition: *preterm* for children born before 37 weeks gestational age, *at term* for children born between 37+0 and 41+6 weeks, and *postterm* for children born equal to or later than 42 weeks gestational age (WHO, 2012).

We defined a developmental problem/delay as (1) one behavioural, cognitive or motor test score in the severe range, or (2) two scores in the moderate range or (3) three scores in the mild range or based on a previous psychiatric diagnosis (i.e., autism spectrum

disorder) or a motor disability confirmed by a specialized health care professional. Classifications of severe, moderate or mild range were as follows: behaviour (severe, ≥70; moderate, 63–69; and mild, 60–62), IQ (severe, <55; moderate, 55–69; and mild, 70–84) and motor performance (severe, <1—cerebral palsy or not able to assess; moderate, ≤5; and mild, 6–7).

Statistics

Characteristics at birth and at one year and four years were described separately for the 'five-years-old cohort', subdivided for singletons and multiples. Fisher's exact test was used for nominal outcome data. A one-sample Kolmogorov–Smirov test was used to determine whether the distribution of the study group's data corresponded with the theoretical distribution in the general Dutch population based on test characteristics, after normal distribution of the study group's data was confirmed by a Q-Q plot. Continuous data were compared using Student's t-test. Statistical analysis was performed using IBM SPSS Statistics 20 (Chicago, IL, USA). A p value of <0.05 was considered significant. Test results of subgroups with children having major birth defects and children having a developmental problem were described separately. We were unable to perform a power analysis on developmental outcomes because of the nature of our study The power analysis was performed soley based on major birth defects reported by questionnaires; we estimated that 190 children were necessary to include in order to detect an increase of five percent of major birth defects compared to a prevalence of four percent in IVF/ICSI (Pinborg, et al., 2004).

Results

Between March 1, 2008, and May 1, 2016, 411 children were live born after TESE-ICSI treatment in Radboudumc (n = 354) and AMC (n = 57), all parents initially consented for follow up of their child before start of the treatment. Four hundred four children entered the follow-up program by returning a questionnaire at birth. We received 319/347 questionnaires from children that has reached the age of one and 146/180 questionnaires from children that has reached the age of four. During our study period 125 children reached the age of five and were invited for assessment.

We completely assessed 89 (71%) children aged between 5 years and 0 months and 5 years and 11 months. Fourteen children (11%) (i.e., their parents) refused to come to the hospital but agreed to fill out questionnaires and provide medical reports from their paediatricians/family doctors. Those 103 completely or partially assessed children formed the 'five-years-old cohort'. Reasons for no follow-up were mainly the burden (n = 7) of the assessment and no response (n = 6).

Table 1 Follow up of live born children after TESE-ICSI

	Total cohort		'Five-years-old cohort'	
	Singletons	Multiples	Singletons	Multiples
Birth	n=321	n=83	n=72/321	n=31/83
Gender				
Boy n (%)	159 (49.5)	43 (51.8)	37 (51.4)	15 (48.4)
Girl n (%)	162 (50.5)	40 (48.2)	35 (48.6)	16 (51.6)
Gestational age mean weeks (SD)	39.5 (2.0)	36.2 (3.0)	39.8 (1.5)	36.0 (3.3)
Preterm n (%)	16 (5.0)	37 (44.6)	4 (5.6)	15 (48.4)
At term n (%)	295 (91.9)	46 (55.4)	63 (87.5)	16 (51.6)
Post term n (%)	10 (3.1)	0	5 (6.9)	0
Birth weight mean gr (SD)	3436 (570)	2403 (608)	3516 (454)	2298 (552)
Major birth defect n (%)	23 (7.2)	1 (1.2)	9 (12.5)	0
Infant death n (%)	2 (0.6)	1 (1.2)	0	0
1 year	n=247	n=72		
Growth parameters				
Weight mean kg (SD)	9.7 (1.2)	9.1 (1.1)	9.8 (1.1)	9.3 (1.1)
Length mean cm (SD)	75.7 (3.1)	74.6 (2.7)	76.1 (3.0)	74.7 (2.2)
Medical & Paramedical history in first year				
Seen by a medical specialist n (%)	85 (34.4)	23 (31.9)	22 (30.6)	13 (41.9)
Underwent surgery n (%)	8 (3.2)	4 (5.6)	4 (5.6)	1 (3.2)
Referral because of developmental issues n (%)	2 (0.8)	0	1 (1.4)	0
Seen by a physiotherapist n (%)	45 (18.2)	20 (27.8)	13 (18.1)	12(40.0)
Hearing problems diagnosed n (%)	1 (0.4)	1 (1.4)	1 (1.4)	0
4 year	n=106	n=38		
Growth parameters				
Weight mean kg (SD)	17.0 (2.1)	16.5 (2.3)	17.1 (2.1)	17.1 (2.0)
Length mean cm (SD)	105 (4.7)	104 (4.5)	105 (4.8)	105 (4.4)
Medical & Paramedical history since 1 years of age				
Seen by a medical specialist n (%)	36 (34.0)	19 (47.5)	27 (37.5)	14 (45.2)
Underwent surgery n (%)	22 (20.8)	9 (23.7)	18 (25.0)	5 (16.1)
Referral because of developmental issues n (%)	5 (4.7)	1 (2.6)	4 (5.6)	0
Seen by a speech therapist n (%)	19 (17.9)	9 (23.7)	13 (18.1)	6 (19.4)
Seen by a physiotherapist n (%)	11 (10.4)	3 (7.9)	8 (11.1)	2 (6.5)
Hearing problems diagnosed n (%)	6 (5.7)	2 (5.3)	5 (7.1)	0
Specialized elementary school needed n (%)	2 (1.9)	1 (2.6)	2 (2.8)	0

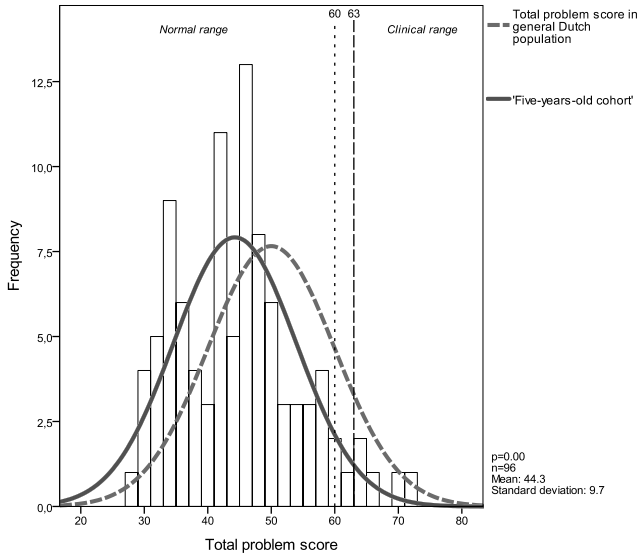
Table 1 Continued

	Total cohort		'Five-years-old cohort'	
	Singletons	Multiples	Singletons	Multiples
5 year	n=72	n= 31		
Growth parameters				
Weight mean kg (SD)	20.8 (2.9)	20.8 (2.8)	20.8 (2.9)	20.8 (2.8)
Length mean cm (SD)	115.3 (4.5)	114.7 (4.7)	115.3 (4.5)	114.7 (4.7)
Medical & Paramedical history last year				
Seen by a medical specialist n (%)	26 (36.1)	7 (22.6)	26 (36.1)	7 (22.6)
Underwent surgery n (%)	9 (12.5)	2 (6.5)	9 (12.5)	2 (6.5)
Referral because of developmental issues n (%)	1 (1.4)	0	1 (1.4)	0
Seen by a speech therapist n (%)	14 (19.4)	9 (29.0)	14 (19.4)	9 (29.0)
Seen by a physiotherapist n (%)	8 (11.1)	3 (9.7)	8 (11.1)	3 (9.7)
Seen by a psychologist n (%)	6 (8.3)	1 (3.2)	6 (8.3)	1 (3.2)
Specialized elementary school needed n (%)	2 (2.8)	0	2 (2.8)	0

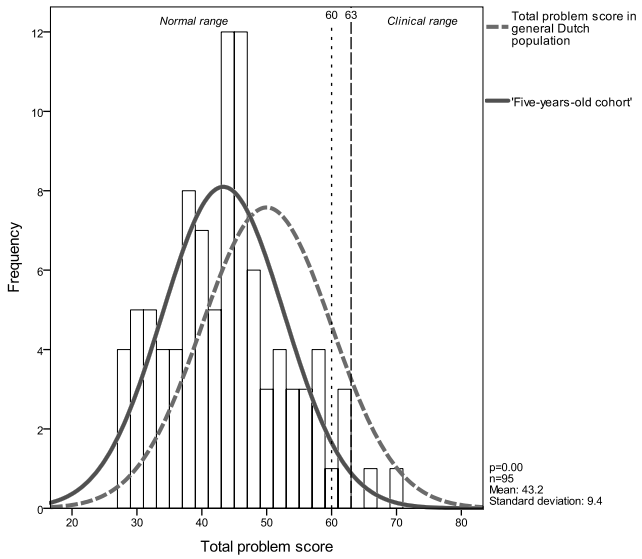
Table 1 shows the baseline characteristics and results of the questionnaires of the total group and of the 'five-years-old cohort'. The mean maternal age at delivery was 32.4±3.8 (range: 23–43) and paternal age 36.8±7.5 (range: 25–64) years. Three infants died in the first week after birth, two singletons were born at 23+1 and 26+1 weeks and one second-born twin was born at 25+1 weeks. There was no significant difference in the prevalence of birth defects (p = 0.23) and percentage of referral because of developmental reasons in 'five-years-old cohort' and the group of children who did not reach the age of five during the study period (p = 1.00).

Results of the behavioural assessment are presented in Figure 1. Parents and teachers of the 'five-years-old cohort' reported significantly less behavioural problems in this group compared with the theoretical distribution in general Dutch children. The total test scores of parents about the behaviour of their children were significantly lower than the scores of the teachers (p = 0.02). While teachers reported significantly more behavioural problems (p = 0.05), mothers reported more externalising problems (p = 0.02) in boys compared with girls. There was no difference in behavioural problems in boys compared with girls reported by questionnaires filled out by fathers. No differences in behavioural performance were found between children born preterm and at term.

Results of the cognitive assessment in terms of total IQ are presented in Figure 2. The 'five-years-old cohort' had a significantly higher IQ compared with the theoretical distribution general Dutch children. Forty-four percent of the mothers and 35% of the fathers reported higher professional education or university levels on the questionnaire at birth of their children. Girls had a significantly higher total IQ (p = 0.04) and processing speed (p = 0.002)

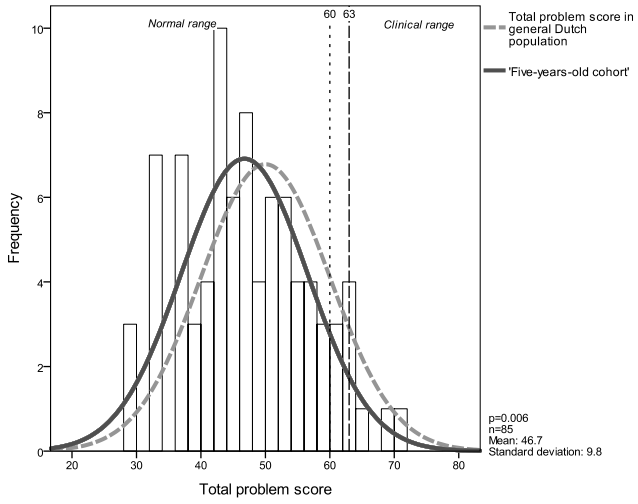


Total problem score was tested by the Child Behavior Checklist reported by the mother.



Total problem score was tested by the Child Behavior Checklist reported by the father.

Figure 1 Behavioural performance in 'five-years-old cohort' reported by the mother, father and teacher



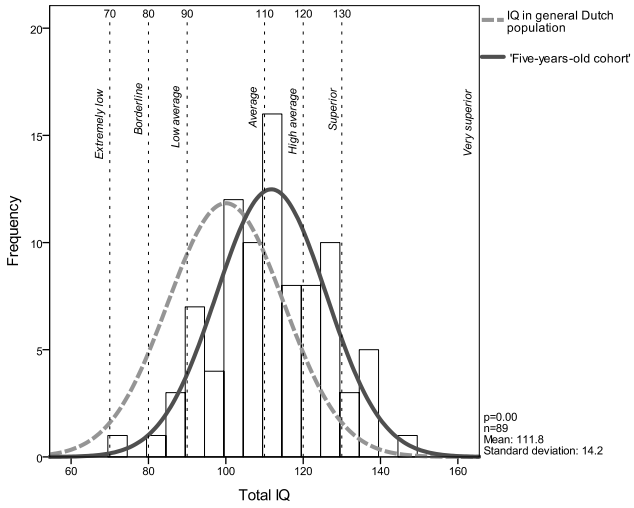
Total problem score was tested by the Teacher Report Form reported by the teacher. Theoretical distribution of test scores in general population was based on a normative value of 50 and a standard devation of 10. A one-sample Kolmogorov-Smirov test was used to determine whether the distribution of the study group's data corresponded with the theoretical distribution in the general Dutch population based on test characteristics.

Figure 1 Continued

compared to boys. Children born at term scored significantly higher for both total (p = 0.002) and performal IQ (p = 0.000) than children born preterm.

Results of the motor assessment are presented in Figure 3. The children in the 'five-years-old cohort' had significantly poorer motor performances compared with the theoretical distribution in general Dutch children. We found 21 (23%) children with a delayed motor performance, 12 of which were at risk of a movement difficulty and 9 had a significant movement difficulty. Nine children already had the support of a paediatric physiotherapist. Total scores (p = 0.006), manual dexterity (p = 0.006) and balance (0.02) were significantly better in girls compared with boys. The scores for children born preterm and at term did not differ statistically with the exception of the component score for balance that showed children born at term had a higher score (p = 0.001).

The results of the assessment of physical development are presented in Table 1. Nine children had a major birth defect: hip dysplasia (ICD-10 Q65.2 and Q65.6), ventricular septal defect (ICD-10 Q21.0), patent ductus arteriosus (ICD-10 Q25.0), galactosemia (ICD-10 E74.2), XYY karyotype (ICD-10 Q98.5), hemivertebra (ICD-10 Q76.4), posterior urethral valves (ICD-10 Q60.4) and inguinal hernia (ICD-10 K40.9). Ninety-six percent of the children had a standard deviation between –2.0 and 2.0 for length and 93% and 95% percent for weight and weight for length, respectively (general population covering 95%) corrected for age and race.



The intelligence quotient (IQ) was tested by the Wechsler Preschool and Primary Scale of Intelligence-III-NL assessed by a paediatric psychologist. Theoretical distribution of test scores in general population was based on a normative value of 100 and a standard devation of 10. A one-sample Kolmogorov-Smirov test was used to determine whether the distribution of the study group's data corresponded with the theoretical distribution in the general Dutch population based on test characteristics.

Figure 2 Cognitive performance in 'five-years-old cohort'

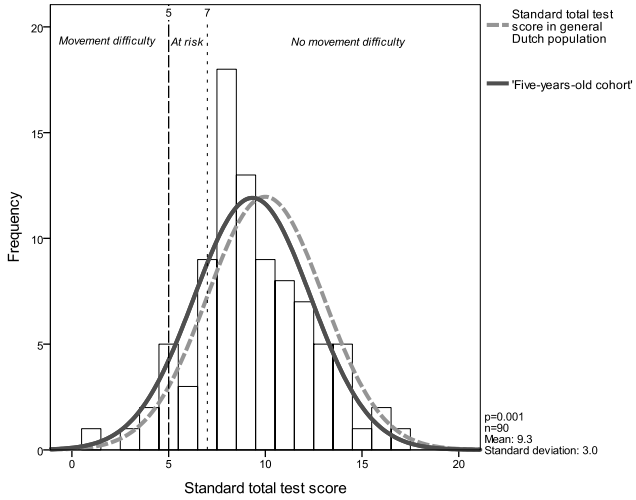
Children with major birth defects

The mean test scores for a subgroup of children with a major birth defect were as follows: behavioural assessment total problem score: 43.0 (mother), 41.4 (father) and 46.5 (teacher); cognitive assessment total IQ, 120.8; and motor assessment standard total test score, 9.6. The scores of this subgroup were in the same range as those of the total 'five-years-old cohort'.

Children with developmental problems

Four children of the 'five-years-old cohort' had developmental problems/delays: Two had a previous psychiatric diagnosis and two presented with two scores in the moderate range. A brief description of those four children follows:

Child 1 was diagnosed with a form of autism (Pervasive Developmental Disorder-Not Otherwise Specified, PDD-NOS). This child was born at term and was diagnosed with posterior urethral valves. After one year he had undergone a renal transplantation because of renal failure, and there was a delay in the developmental milestones. At four years he had deprived language and motor development and pubertas praecox; he attended a specialized primary school.



The standard total test score was tested by the second Dutch version of the Movement Assessment Battery for Children, assessed by a paediatric physical therapist. Theoretical distribution of test scores in general population was based on a normative value of 10 and a standard devation of 3. A one-sample Kolmogorov–Smirov test was used to determine whether the distribution of the study group's data corresponded with the theoretical distribution in the general Dutch population based on test characteristics.

Figure 3 Motor performance in 'five-years-old cohort'

Child 2 was also diagnosed with PDD-NOS. He was born at term. At one year he visited a paediatrician because of reflux. At four years he had deprived language and motor development, and he had undergone surgery (placing tympanostomy tubes and a tonsillectomy).

Child 3 was diagnosed with XYY syndrome during pregnancy by a chorionic villus sampling. He was born at term. At one year he had developed normally, but at four years he had deprived language and motor development and attended a specialized primary school. At five years this boy had developmental problems suspicious for autism spectrum disorder, but he had not been finally diagnosed. The teacher reported behavioural problems in the clinical range (total problem score of 65). Moreover, this child had deprived language development. For this reason we were unable to assess cognitive performance. This child also had a significant movement difficulty (standard total test score: 1).

Child 4 was born at term. At one year and four years his weight was above average (+2.2 SD and +2.1 SD) with no other remarks. At five years the teacher reported his behaviour in the borderline range (total problem score of 60), whereas the parents did not. This child had an IQ of 72 and was at risk for a movement difficulty (standard total test score of 7).

In addition to those four children with developmental problems/delays, two other children did not meet our definition of having a developmental problem. A brief description of those two children follows:
Child 1 had internalizing behavioural problems within the clinical range, according to the mother, and total behavioural problems in the clinical range, according to the teacher, but the father reported none. The IQ was 139, and he had a normal motor performance. The parents suspected an autism spectrum disorder, but this child has not yet been diagnosed.
Child 2 had behavioural problems in the clinical range, according to the parents; the teacher did not fill out the questionnaire. The child had a short stature (length SD 2.5; weight SD 2.4). His IQ was 129, and he had a normal motor performance.

Discussion

In this prospective study we examined the development of children born after TESE-ICSI. We evaluated the development of 404 children born after TESE-ICSI at birth and at one year and four years of age, and we evaluated the development of 103 five-year-old children with behavioural, cognitive and motor performance tests and growth parameters.
Our 'five-years-old cohort' scored significantly better on behavioural and cognitive performance but significantly worse on motor performance than the general population. We found four children (3.8%) of the 'five-years-old cohort who presented with developmental problems. Our results concerning the safety of TESE-ICSI regarding its long-term effects on development and health in children born after these procedures seem reassuring.
In this study we found two children with a form of autism, which is a relatively high prevalence in this small sample. In the general population the prevalence of autism is about one percent in the age group between four and seven year old (CBS, 2014). Although it is known that autism is more common in children of older fathers (D'Onofrio, et al., 2014), the fathers of the two children were 28 and 33 years at child birth.
Our findings of the cognitive performance in our 'five-years-old cohort' of boys and girls and pre- or at-term-born children were in accordance with the literature (Hoff Esbjorn, et al., 2006, Strauss, et al., 2006). Moreover, our results are in line with a systematic review regarding IVF children, which concludes that, in general, long-term mental and emotional health outcomes from IVF treatment are reassuring (Hart and Norman, 2013). Punamäki et al. recently studied the mental health and developmental outcomes of 225 children, 7–8 years old, born after IVF/ICSI and compared the outcomes with 278 naturally conceived controls (Punamaki, et al., 2016). They found that children born after IVF/ICSI did not differ with regard to mental health or social and cognitive developmental problems. They did not specify whether they included children born after TESE-ICSI, and their results were based only on parental reports. In literature, generally CBCL scores are higher than TRF scores on most scales, this is in discordance with our study (Rescorla, et al., 2014).

In our study we found a better behavioural and cognitive performance of children born after TESE-ICSI compared with the general Dutch population. However, this may be related to a high education level of the children's parents. A prospective follow-up study of four-year-olds born to infertile couples (including 63 IVF, 53 natural cycle IVF, and 79 natural conception children) found no causal effect between ovarian stimulation or in vitro procedures and cognitive and behavioural outcomes. But the authors concluded, based on the results of IQ tests and CBCL questionnaires, that infertility per se, and especially more severe infertility, negatively affects children's cognitive and behavioural outcomes (Schendelaar, et al., submitted - 2015). They expressed the severity of infertility in terms of time to pregnancy rather than by specific underlying causes. However, in couples with males suffering from azoospermia, time to pregnancy may not be a good parameter because their failure to conceive is not dependent on time of exposure.

We found that motor performance in our 'five-years-old cohort' was significantly worse compared with the general Dutch population. That might be due to a higher proportion of multiples and preterm births in our cohort compared with the general population. However, in our cohort we found no difference in total scores in children born preterm or at term, maybe because moderately preterm infants born between 32 and 37 weeks were included. Although there is a significant difference compared with the general Dutch population, the mean total test score of our cohort is still within the normal range and less clinically relevant. However, it is striking that we referred four children for physical therapy. One child's parents were advised to stop physical therapy, and another child's parents to restart physical therapy. In our study children born at term had a better score for balance compared with children born preterm, which is in line with what is known from the general population (de Kieviet, et al., 2009).

A major birth defect was observed in 5.9% of the children in the total cohort and 8.7% of the children in our 'five-years-old cohort', this percentage is in line with previous studies (Belva, et al., 2011, Woldringh, et al., 2010) regarding ICSI outcomes with non-ejaculated sperm. The prevalence of major birth defects in the general population in the Netherlands in 2010-2012 was estimated between 3.6-3.7% (TNO, 2014). It is known that the prevalence of major birth defect after IVF/ICSI is elevated compared with general population. A systematic review showed that the use of testicular sperm doesn't contribute to an increased risk compared with ejaculated sperm (Woldringh, et al., 2010) . We previously studied the probability of achieving a live birth in TESE-ICSI in a cohort of azoospermic males and their female partners (Meijerink, et al., 2016), the parents of the children in our current study were part of that cohort. In this cohort, the majority (85%) of the TESE-ICSI cycles were performed with frozen thawed testicular sperm. ICSI results were as follows: mean number of oocytes retrieved 10.5; mean number of fertilized 2pn embryos 4.1; mean number of transferred embryos per cycle 1.3. In this cohort 22% of the cycles resulted in a live birth.

The strength of our study is its prospective longitudinal design with limited bias. In the Netherlands, because of a moratorium effective until 2014, ICSI with testicular retrieved

spermatozoa was allowed only in research settings. For that reason all children born from couples who underwent TESE-ICSI treatment in the Netherlands were included in this study, which limited bias. Furthermore, our lost to follow-up during our study was limited to 18%.

Despite the prospective nature and the nationwide character of our study, there are some limitations. First, we were unable to compare our cohort with naturally conceived controls. To compare our 5-years-old cohort with the naturally conceived children we used standard deviations and the range of test scores. Second, we were unable to perform a power analysis on developmental outcomes because of the nature of our study. Moreover, there were some children lost to follow up which may attrition bias our study. However, we did not find any underlying reasons for non-participation potentially related to the health of the children. Finally, data may be influenced by interobserver variations. The cognitive assessment was administered by five paediatric psychologists; the motor assessment by three paediatric physical therapists (two of the three therapists assessed about half of the children and both therapists were trained by the third therapist); and the physical examination by one physician.

In conclusion, we found that the behavioural and cognitive performance scored better but the motor performance was poorer in five-year olds born after TESE-ICSI compared with the general population. Despite statistical significance, differences in outcomes compared with the general population were small and of questionable or clinical importance, being small in magnitude, subject to influence of small numbers of measures at the extremes of rang, and loss of follow up of some subjects. Therefore, the long-term effects on development and health in children born after TESE-ICSI seem reassuring. Our findings could empower health-care providers to comprehensively counsel couples who consider TESE-ICSI treatment and help them in their concern about their children's development. We want to emphasize that follow-up of these children should continue because knowledge of their long-term health is scarce.

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Supplemental Table 1 Classification of developmental subtests

	Severe	Moderate	Mild	Normal
Behavioural performance (total problem score)	≥70	63-69	60-62	<60
Cognitive performance (total intelligence quotient)	<55	55-69	70-84	≥85
Motor performance (total test score)	<1/not possible to assess/CP	≤5	6-7	>7

A developmental problem/delay was defined by us as [1] one behavioural; cognitive or motor test score in severe range, or [2] two scores in moderate range or [3] three in mild range, or based on previous psychiatric diagnosis (i.e. autism spectrum disorder) or motor disability confirmed by a specialized health care professional. CP= cerebral palsy.

Supplemental Table 2 Assessment of behavioural performance 'five-years-old cohort'

Child behaviour[1]	Mother n=96	p-value	Father n=95	p-value	Teacher n=85	p-value
Total score mean (SD)	44.3 (9.7)	0.00	43.2 (9.4)	0.00	46.7 (9.8)	0.006
Normal range (<60) n (%)	89 (92.7)		90 (94.7)		75 (88.2)	
Borderline range (60-62) n (%)	2 (2.1)		3 (3.2)		6 (7.1)	
Clinical range (≥63) n (%)	5 (5.2)		2 (2.1)		4 (4.7)	
Internalizing problems score mean (SD)	45.5 (10.4)	0.00	45.0 (9.9)	0.00	46.3 (10.4)	0.001
Normal range (<60) n (%)	87 (90.6)		84 (88.4)		77 (90.6)	
Borderline range (60-62) n (%)	3 (3.1)		8 (8.4)		3 (3.5)	
Clinical range (≥63) n (%)	6 (6.3)		3 (3.2)		5 (5.9)	
Externalizing problems score mean (SD)	45.3 (10.4)	0.00	44.9 (9.2)	0.00	47.9 (8.4)	0.024
Normal range (<60) n (%)	86 (89.6)		90 (94.7)		76 (89.4)	
Borderline range (60-62) n (%)	4 (4.2)		1 (1.1)		5 (5.9)	
Clinical range (≥63) n (%)	6 (6.3)		4 (4.2)		4 (4.7)	

1. Child Behaviour Checklist (CBCL) reported by parents, and Teacher Report Form (TRF) reported by the teacher

Supplemental Table 3 Assessment of cognitive performance 'five-years-old cohort'

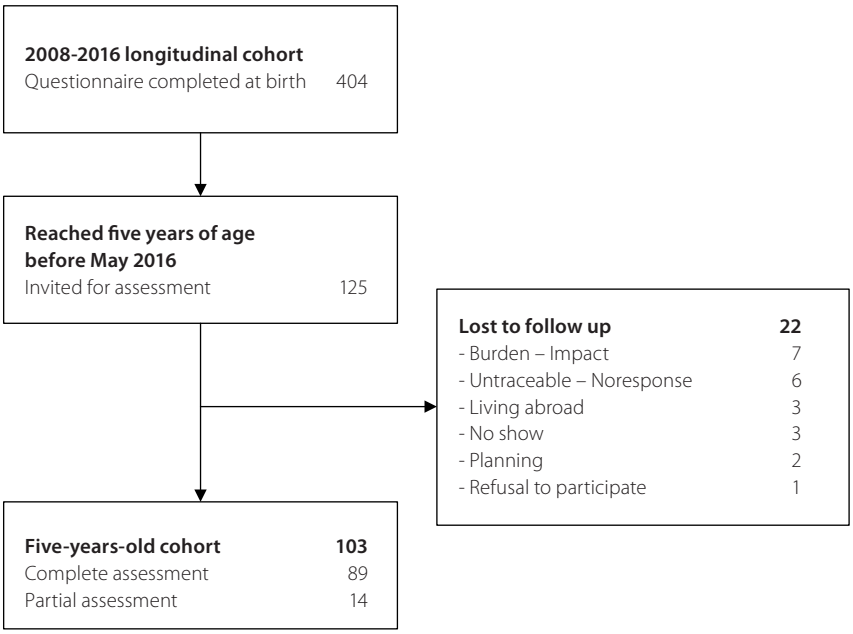
Child cognitive performance[1]	n=89	p-value
Total Intelligence Quotient mean (SD)	111.8 (14.2)	0.000
Very superior (≥130) n (%)	9 (10.1)	
Superior (120-129) n (%)	18 (20.2)	
High average (110-119) n (%)	24 (27.0)	
Average (90-109) n (%)	33 (37.1)	
Low average (80-89) n (%)	4 (4.5)	
Borderline (70-79) n (%)	1 (1.1)	
Verbal Intelligence Quotient mean (SD)	111.6 (14.2)	0.000
Very superior (≥130) n (%)	10 (11.2)	
Superior (120-129) n (%)	15 (16.9)	
High average (110-119) n (%)	23 (25.8)	
Average (90-109) n (%)	36 (40.4)	
Low average (80-89) n (%)	2 (2.2)	
Borderline (70-79) n (%)	2 (2.2)	
Missing	1 (1.1)	
Performal Intelligence Quotient mean (SD)	110.3 (11.9)	0.000
Very superior (≥130) n (%)	5 (5.6)	
Superior (120-129) n (%)	15 (16.9)	
High average (110-119) n (%)	27 (30.3)	
Average (90-109) n (%)	37 (41.6)	
Low average (80-89) n (%)	5 (5.6)	
Borderline (70-79) n (%)	0	
Processing speed mean (SD)	100.5 (14.4)	0.576

1. Wechsler Preschool and Primary Scale of Intelligence – III NL (WPSSI-III-NL) assessed by a paediatric psychologist

Supplemental Table 4 Assessment of motor performance 'five-years-old cohort'

Child motor performance[1]	Preterm n= 16	At term n= 74	p-value for total group n=90
Standard total test score mean (SD)	9.5 (3.1)	9.3 (3.0)	0.001
Normal range (>7) n (%)	12 (75)	57 (77)	
At risk (6-7) n (%)	1 (6)	11 (15)	
Movement difficulty (≤5) n (%)	3 (19)	6 (8)	
Manual dexterity mean (SD)	9.5 (2.5)	9.9 (2.8)	0.013
Normal range (>7) n (%)	13 (82)	61 (82)	
At risk (6-7) n (%)	2 (13)	9 (12)	
Movement difficulty (≤5) n (%)	1 (6)	4 (5)	
Aiming & catching mean (SD)	8.7 (3.6)	9.4 (2.6)	0.000
Normal range (>7) n (%)	10 (63)	54 (73)	
At risk (6-7) n (%)	2 (13)	15 (20)	
Movement difficulty (≤5) n (%)	4 (25)	5 (7)	
Balance mean (SD)	10.3 (2.8)	8.9 (2.8)	0.000
Normal range (>7) n (%)	13 (82)	50 (68)	
At risk (6-7) n (%)	3 (19)	17 (23)	
Movement difficulty (≤5) n (%)	0	7 (9)	

1. Movement Assessment Battery for Children 2 –NL (MABC-2-NL) assessed by a paediatrics physiotherapist, results sub dived for children born preterm (i.e. <37 weeks of gestation) and children born at term (i.e. ≥37 weeks of gestation including post term).



'Five-years-old cohort': all children born after TESE-ICSI procedures in Rumc and AMC, who were completely assessed for behavioural, cognitive, motor and/or physical performance or partially assessed by questionaires for medical condition and behaviour, or providing information from a general practioner or pediatrian, at the age of five.

Supplementary figure Flowchart of the included live born children after TESE-ICSI

6

Influence of paternal age on ongoing pregnancy rate at eight weeks' gestation in assisted reproduction

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Abstract

A retrospective cohort study was performed with the followings aims: to evaluate the influence of paternal age on best embryo quality available for embryo transfer on the third day; biochemical pregnancy rate; miscarriage rate and ongoing pregnancy rate at 8 weeks' gestational age, after IVF or intracytoplasmic sperm injection (ICSI) treatment, respectively, including treatment with non-ejaculated spermatozoa. In total, 7051 first IVF/ICSI cycles in Radboud university medical center, between 1 January 2001 and 1 June 2013 were included in this study. A statistical model was used to analyse the effect of paternal age and maternal age. No statistically significant differences between the paternal age groups were found with respect to the probability of an ongoing pregnancy after the first cycle (35–44 years: odds ratio [OR] = 0.97 [95% confidence interval [CI]: 0.86 to 1.10] and ≥45 years: OR = 1.01 [95% CI: 0.82 to 1.26]), respectively, compared with <35 years of age (control). Similar results were found with respect to paternal age and the availability of a top quality embryo for transfer, biochemical pregnancy and miscarriage. However, live birth was not taken into account. In conclusion, paternal age did not affect ongoing pregnancy rates in first IVF/ICSI cycles.

Introduction

In Western society and developed countries, there is a tendency for delayed parenthood as a consequence of social economical welfare, personal education development, increased life expectancy, divorce and restarting new families. Nowadays, not only women are delaying parenthood; men's ages for parenthood have also increased in the last decade. In the Netherlands in 2000, 11.1% (n = 22,981) of the children born had a father over 40 years old; in 2012 this percentage was 16.4% (n = 28,888) (CBS, 2011).

While for women their biological clock will determine the end of their fecundity, men's biological clocks do not play such a prominent role, as men can produce spermatozoa until a very advanced age. Currently, there are no legal or biological restrictions given to participation of elderly men in assisted reproductive programmes. The focus on factors affecting the outcome of assisted reproductive techniques is mainly related to the influence of female factors, including age, diagnosis, co-morbidity and ovarian stimulation. It is well known that women older than 35 years have a higher risk of spontaneous abortion, pregnancy complications and chromosomal abnormalities, but couples with men over 40 years of age also seem to have a higher risk for miscarriage (de la Rochebrochard and Thonneau, 2002). Another study found a 4.5-fold higher risk of having a child with trisomy 21 in men over 45 years of age compared with men younger than 30 years (Zhu, et al., 2005). They suggest that this could be induced by biological or environmental factors causing gamete mutations in men. It seems that reproductive function in both women and men declines with age (Wiener-Megnazi, et al., 2012).

Fathers in couples undergoing assisted reproductive techniques are older than their fertile counterparts (Stern, et al., 2014), but only limited or controversial results about the influence of paternal age on reproductive outcome after assisted reproductive techniques have been published. Previous studies show discordant findings in terms of effects on embryo quality, pregnancy rate and live born delivery (de La Rochebrochard, et al., 2006, Ferreira, et al., 2010, Klonoff-Cohen and Natarajan, 2004). In these studies the use of non-ejaculated spermatozoa (of epididymal or testicular origin) was not included. However, older men who had a previous vasectomy, in particular, are candidates for intra-cytoplasmic sperm injection (ICSI) with non-ejaculated spermatozoa for assisted reproductive treatment. Studies using an ovum donation model reveal that embryo implantation rates decline with increasing paternal age, but show no agreement regarding pregnancy outcome and have not been adjusted for recipient age (Humm and Sakkas, 2013, Robertshaw, et al., 2014). The aim of this study is to look at the influence of paternal age on best embryo quality available for embryo transfer (ET), biochemical pregnancy rate (BPR), miscarriage rate and ongoing pregnancy rate (OPR) up to 8 weeks' gestational age, after IVF or ICSI treatment, respectively, including treatment with non-ejaculated spermatozoa. To this end, a large retrospective cohort study of first assisted reproductive treatment cycles was performed.

Materials and methods

Study population

A retrospective cohort study was performed to investigate the influence of paternal age on reproductive outcome when using assisted reproductive techniques. Data were collected for all first cycles of assisted reproductive treatment at the Radboud university medical center (Radboudumc) between 1 January 2001 and 1 June 2013. Data were obtained with respect to: the age of men and women at the time of oocyte retrieval; type of assisted reproductive technique (IVF or ICSI); type of spermatozoa used for the treatment (ejaculated or non-ejaculated: percutaneous epididymal sperm aspiration [PESA]; micro-surgical epididymal sperm aspiration [MESA] or testicular sperm extraction [TESE]); condition of spermatozoa (fresh or cryopreserved); FSH dose; number of follicles and oocytes; fertilization rate; embryo quality of transferred embryos; single embryo transfer (SET) or double embryo transfer (DET); number of good quality embryos for cryopreservation; and pregnancy results (biochemical and ongoing pregnancies).

Of all first cycles (n = 7246), the study excluded couples who underwent assisted reproductive treatment in a modified natural cycle (n = 51); women who did not have ET for risk of ovarian hyperstimulation syndrome (OHSS); or assisted reproductive treatment in oncology patients because of fertility preservation (n = 74) or for oocyte vitrification (n = 70). As a result, 7051 first assisted reproductive technique cycles were included in this study. Ethical committee approval was not required for this retrospective study according to the Medical Treatment Agreements Act (Medical Treatment Agreements Act, 1994),

Procedures

Patients underwent assisted reproductive treatment in a long agonist protocol as described previously by Dam *et al.* (Dam, et al., 2012). Briefly, stimulation was initiated by injections with FSH with doses depending on anti-Müllerian hormone concentrations and/or antral follicle count. Oocyte retrieval was planned when ≥3 follicles with a diameter of ≥17 mm were observed by ultrasound examination, and was performed 36 h after human chorionic gonadotrophin (HCG) injection. ICSI was performed as described previously by Palermo *et al.* (Palermo, et al., 1992).

Luteal phase support was started on the day of oocyte retrieval by vaginal administration of 200 mg progesterone (Utrogestan) three times daily, continued until pregnancy test.

Three days after oocyte retrieval, one or two embryos were transferred. SET or DET was performed depending on national policy, the woman's age, the woman's medical history and the couple's preference. Transfer policy has changed over time, the last 3 years into SET in women <38 years in the first two IVF/ICSI cycles including cryotransfers. No embryo transfer was performed in cases of absence of fertilization, abnormal embryos or in cases of (risk for) OHSS.

Embryo scoring and evaluation was based on fragmentation and the number of blastomeres using an inverted light microscope by a qualified embryologist or laboratory

technician. All embryos were examined to determine fragmentation and the number of blastomeres on day 3 (ESHRE, 2000). The quality of embryos selected for transfer was classified from best to lowest as follows: A-quality embryos with 0–20% fragmentation and containing seven, eight or nine blastomeres (top quality); B-quality embryos with 20–50% fragmentation containing seven or eight blastomeres, or embryos with 0–20% fragmentation containing four, five or six, or ten, eleven or twelve blastomeres (mean quality); C-quality embryos (poor quality). After scoring, one or two of the best embryo(s) available were selected for embryo transfer. Fifteen days after ET, patients performed a urine pregnancy test at home and informed the hospital about whether or not a biochemical pregnancy had occurred. A miscarriage was defined as a pregnancy diagnosed only by the detection of HCG in serum or urine and that did not develop into a clinical pregnancy or the spontaneous loss of a clinical pregnancy that occurred before 20 completed weeks of gestational age (Zegers-Hochschild, et al., 2009). A clinical pregnancy was defined as a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy. It includes ectopic pregnancy (Zegers-Hochschild, et al., 2009). An ongoing pregnancy was defined as the appearance of a foetal heartbeat examined by ultrasound at 8 weeks' gestational age.

Statistical analysis

Descriptive statistics are used to present the characteristics by paternal age group. Three (different) age classes were used for men and women based on literature (de La Rochebrochard, et al., 2006, de La Rochebrochard and Thonneau, 2003, Wiener-Megnazi, et al., 2012). Multivariable logistic regression was used to study the influence of paternal and maternal age on the probability after the first cycle of an ongoing pregnancy, of a biochemical pregnancy and of an A-quality embryo and a miscarriage separately. The dependant variable was an ongoing pregnancy (a biochemical pregnancy, an A-quality embryo, a miscarriage, respectively). The independent class variables were paternal age (3 levels: < 35, $35 - 44$, ≥ 45 years) and maternal age (3 levels: < 35, $35 - 39$, ≥ 40 years). The type of assisted reproductive techniques and embryos transferred (none, SET, DET) were treated as confounder variables. Initially, the interaction between paternal age and maternal age was also included in the model to evaluate whether or not this contributed to the fit of the model. Finally, a confirmative analysis was performed using the initial model and the data related to SET or DET only. The adjusted odds ratios (OR) with the appropriate 95% confidence interval (CI) of the final models are presented.

In an analogous manner to the methods described above, logistic regression analysis was used to study the influence of paternal age using the data of IVF, ICSI and ICSI with non-ejaculated spermatozoa only and of paternal age groups ≥50 and ≥60 years. Statistical analysis was performed using IBM SPSS Statistics 20 for Windows (IBM Inc., USA) and SAS version 9.2 for Windows (SAS Institute, Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant.

Results

In total, 16,343 assisted reproductive techniques cycles were performed between 1 January 2001 and 1 June 2013 in Radboudumc. Of these, 7246 were related to a first assisted reproductive treatment cycle of unique couples. Cycles with oocyte vitrification, no ET because of OHSS or fertility preservation and modified natural cycles, were excluded. As a result, 7051 assisted reproductive techniques cycles were included in this study (Figure 1).

Table 1 Baseline characteristics and ongoing pregnancy rate by paternal age group

	Paternal age (year)					
	<35		35-44		≥45	
	n	median (range)/ n (%)	n	median (range)/ n (%)	n	median (range)/ n (%)
Maternal age (year)	3076	30.7 (20-42)	3339	35.3 (21-44)	636	36.8 (22-43)
Paternal age (year)	3076	32.0 (17-34)	3339	38.3 (35-44)	636	48.6 (45-73)
First ART cycles	3076		3339		636	
IVF		1548 (50.3)		1971 (59.0)		233 (36.6)
ICSI		1528 (49.7)		1368 (41.0)		403 (63.4)
ejaculated sperm		1202 (39.1)		956 (28.6)		163 (25.6)
non-ejaculated sperm		326 (10.6)		412 (12.3)		240(37.7)
Fertilisation		66.7 (0-100)		66.7 (0-100)		66.7 (0-100)
No. of embryos transferred	3076	2 (0-2)	3339	2 (0-2)	636	2 (0-2)
No ET		270 (8.8)		313 (9.4)		66 (10.4)
SET		1253 (40.7)		1252 (37.5)		236 (37.1)
DET		1553 (50.5)		1774 (53.1)		334 (52.5)
≥1 A-quality embryo		1642 (53.4)		1678 (50.3)		305 (48.0)
Ongoing pregnancy rate		853 (27.7)		792 (23.7)		150 (23.6)

ART: assisted reproductive techniques IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; Fertilisation: percentage of inseminated (or injected) oocytes fertilised in IVF (or ICSI); ET: embryo transfer; SET: single embryo transfer; DET: double embryo transfer; A-quality embryo: embryos with 0-20% fragmentation and containing 7, 8 or 9 blastomeres on the 3th day after ovum pick up.

In 776 cycles, assisted reproductive treatment was performed with cryopreserved spermatozoa: 17 cycles in the IVF group, 111 cycles in the group of ICSI with ejaculated spermatozoa and 648 in the group of ICSI with non-ejaculated spermatozoa. The group with ICSI with non-ejaculated spermatozoa consisted of: 570 PESA-ICSI, 19 MESA-ICSI and

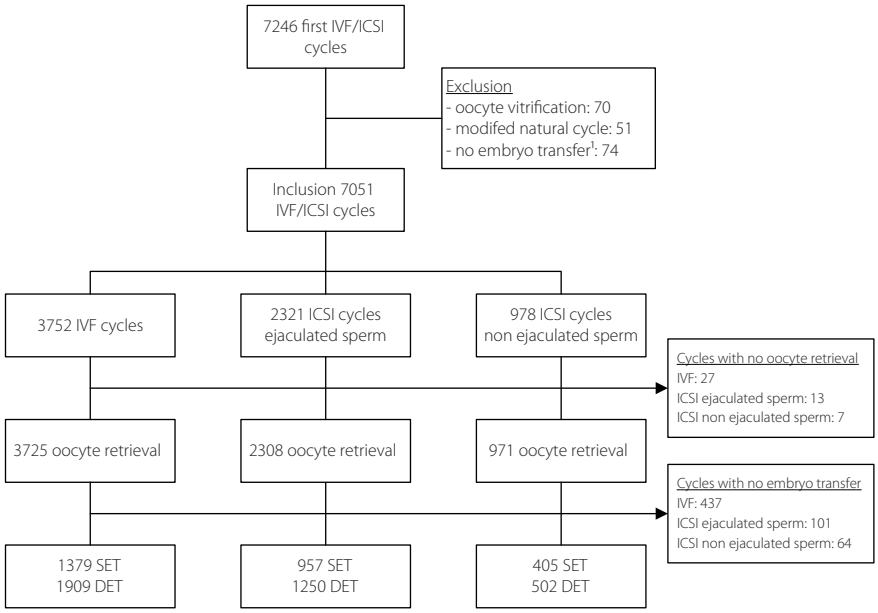

Figure 1 Flow diagram of all patients included in this study

389 TESE-ICSI cycles. For those cycles with cryopreserved spermatozoa, men's age was considered as that at the time of cryopreservation.

In Table 1 the baseline characteristics and ongoing pregnancy rate are presented according to paternal age group. IVF was the most frequent treatment in the two youngest paternal age groups, whereas in the eldest group ICSI with non-ejaculated spermatozoa was as frequently applied as IVF. This study contained 636 men of ≥45 years; the eldest man in this study had an age of 73 years. The ongoing pregnancy rate was 27.7% (853/3076), 23.7% (792/3339) and 23.6% (150/636) in the youngest, intermediate and eldest paternal age group, respectively.

The interaction term between paternal age and maternal age never statistically significantly improved the fit to the data (likelihood-ratio test) and is therefore omitted from the models presented. In addition, a significant relationship between number of embryos transferred and paternal age group was not found. Therefore, the variable number of embryos transferred was omitted from the final model. In Table 2 the adjusted OR of paternal and maternal age for the probability of ongoing pregnancy and for the probability biochemical pregnancy and of the availability of at least one A-quality embryo, respectively, are presented. The OR's are adjusted for the type of treatment and the other

Table 2 Adjusted OR with 95% confidence interval (CI) of paternal age and of maternal age for the probability of an A-quality embryo, biochemical and ongoing pregnancy after a first ART cycle

		A-quality embryo	Biochemical pregnancy	Ongoing pregnancy
	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Maternal age (year)				
<35	4526	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-39	2081	0.77 (0.69-0.87)	0.70 (0.62-0.80)	0.70 (0.61-0.80)
≥40	444	0.70 (0.57-0.86)	0.47 (0.37-0.60)	0.43 (0.32-0.57)
Paternal age (year)				
<35	3076	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-44	3339	1.00 (0.90-1.11)	0.98 (0.87-1.10)	0.97 (0.86-1.10)
≥45	636	0.90 (0.75-1.09)	1.02 (0.83-1.24)	1.01 (0.82-1.26)

OR: odds ratio, ART: assisted reproductive technique, A-quality embryo: embryos with 0-20% fragmentation and containing 7, 8 or 9 blastomeres on the 3th day after ovum pick up.
Note that the adjusted OR's are adjusted for type of treatment and the other variable in model by using multivariable logistic regression analysis.

variable in the model. No statistically significant differences between the paternal age groups were found with respect to the probability of an ongoing pregnancy after the first cycle (35–44 years: OR = 0.97 [95% CI: 0.86 to 1.10] and ≥45 years: OR = 1.01 [95% CI: 0.82 to 1.26], respectively, compared with <35 years of age). Similar results were found with respect to paternal age and the probability of the availability of at least one A-quality embryo.

In addition, the probability of an ongoing pregnancy after the first cycle in the (middle) maternal age group of 35 to 39 years was significantly lower compared with the younger group <35 years, OR = 0.70 (95% CI: 0.61 to 0.80) and significantly higher compared with the oldest group ≥40 years, OR = 1.63 (95% CI: 1.22 to 2.17). These differences were not significantly different between the paternal age groups. The study did not find a statistically significant paternal age effect in subgroups with a paternal age, respectively ≥50 (*n* = 244) and ≥60 (*n* = 29) years (data not shown). Similar results were found with respect to maternal age and the availability of at least one A-quality embryo after the first cycle, except that the probability in the two older maternal age groups were similar but significantly lower compared with the youngest age group: 35–39 years OR 0.77 (95% CI: 0.69–087) and ≥40 years OR 0.70 (95% CI: 0.57–0.86). The study then looked at the influence of the paternal and maternal age in the subgroup of first cycles related to IVF, ICSI and ICSI with non-ejaculated spermatozoa specifically and found nearly identical results as presented above (Supplementary Tables 1-3).

Table 3 Adjusted OR with 95% confidence interval (CI) of paternal age and of maternal age for the probability of a miscarriage after detecting a biochemical pregnancy after a first ART cycle

		IVF		ICSI ejaculated sperm		ICSI non-ejaculated sperm		Total group
	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)	n	OR (95% CI)
Maternal age (year)								
<35	742	1.00 (reference)	702	1.00 (reference)	255	1.00 (reference)	1699	1.00 (reference)
35-39	363	1.40 (1.02-1.94)	164	1.05 (0.69-1.60)	82	0.76 (0.40-1.45)	609	1.17 (0.93-1.48)
≥40	73	1.69 (0.98-2.91)	14	1.70 (0.54-5.36)	9	2.13 (0.54-8.45)	96	1.65 (1.04-2.59)
Paternal age (year)								
<35	524	1.00 (reference)	472	1.00 (reference)	130	1.00 (reference)	1126	1.00 (reference)
35-44	586	0.95 (0.69-1.29)	349	1.09 (0.77-1.53)	138	1.10 (0.61-1.97)	1073	1.02 (0.83-1.27)
≥45	68	0.98 (0.54-1.77)	59	0.97 (0.50-1.90)	78	1.18 (0.59-2.36)	205	1.01 (0.70-1.45)

OR: odds ratio; ART: assisted reproductive technique.
Note that the adjusted OR's are adjusted for type of treatment and the other variable in model by using multivariable logistic regression analysis.

In Table 3 the adjusted OR of paternal age and of maternal age for the probability of a miscarriage after detecting a biochemical pregnancy are presented. No statistically significant differences between the paternal age groups were found. However, the probability of a miscarriage after the first cycle in the maternal age group of ≥40 years was significantly higher compared with the youngest group <35 years, OR 1.65 (95% CI: 1.04–2.59).

Discussion

The results from this study show a significant negative influence of maternal age on ongoing pregnancy using assisted reproductive techniques, as expected (Templeton, et al., 1996), and moreover a higher chance of miscarriage. However, no paternal age effect was evident in these two outcomes. In addition, no paternal ageing effect on best embryo quality available for transfer and biochemical pregnancy was found in this study. In subgroup analyses based on IVF, ICSI and ICSI with non-ejaculated spermatozoa, nearly identical results were found.

A French retrospective cohort study found evidence for a paternal age effect on failure to conceive linked to male ageing (de La Rochebrochard, et al., 2006). However, they only included IVF cycles in which the female partners were sterile due to bilateral tubal obstruction or absence of both tubes. If there is a paternal age effect, it will be found most clearly in the group of couples with young women and older men (i.e. men ≥40 years). They found no significant paternal ageing effect on failure to conceive in this group (OR 1.25, 95% CI 0.43–3.62), but mentioned that the group in the study was very small ($n = 22$) so their results should be treated with caution (de La Rochebrochard, et al., 2006).

For the present study the total group of men aged ≥40 years is much larger ($n = 1676$ versus $n = 480$) compared with that of de La Rochebrochard *et al*., so a more reliable interpretation is possible. It should be noted that in the eldest paternal age group ICSI treatment was used in 63.4% of the cases, while in the younger group this was 49.7%. Besides, it has to be noticed that de la Rochebrochard *et al*. did not give a definition of an ongoing pregnancy and their outcome measure was failure to conceive instead of ongoing pregnancy rate as in this study.

A systematic review of 10 studies with assisted reproductive treatment outcomes found an age-dependent decrease in semen volume, but other parameters of semen analysis were not affected (Dain, et al., 2011). Furthermore, nine of them concluded no significant paternal age effect on assisted reproductive treatment outcome. An important limitation mentioned was the poorly described and small group of advanced paternal age (>50 years) in a number of studies (Dain, et al., 2011). The present study included 636 males ≥45 years and 244 men equal or above ≥50 years. At this moment, as far as we are aware, no large studies concerning the influence of paternal age in assisted reproductive treatment with non-ejaculated spermatozoa have been published. Although the data were limited for this subgroup, the results of this subgroup analysis were comparable with the results of the IVF and ICSI groups with ejaculated spermatozoa.

The strength of this study is the large number of first assisted reproductive treatment cycles available for data analysis, especially in the eldest category. This enabled us to analyse subgroups, especially ICSI with non-ejaculated spermatozoa. A larger share of ICSI with non-ejaculated spermatozoa in the eldest male group could probably be explained by restarting new families after failed vasovasostomy. Those couples have an indication for ICSI with epididymal or testicular spermatozoa.

This study has some limitations. It lacks information on live births. Therefore, ongoing pregnancy at a gestational age of 8 weeks was chosen as the major outcome measure. Information on live births would be useful for analysis of the effect of paternal age on this outcome measure. The influence of men's lifestyle factors and congenital abnormalities in relation to their fertility were also not taken into account. However, the effects of paternal age on miscarriage were analysed. During the 13-year study period, changes and improvements have taken place in assisted reproductive techniques which might influence the study outcome. However, despite the long study period, the ongoing

pregnancy rate had some fluctuations but did not suggest an increase in ongoing pregnancy rate over time (data not shown).

Spermatozoa quality seems to decrease with age (Sharma, et al., 2015), resulting in decline in semen volume, percentage motility, progressive motility, normal morphology and unfragmented cells (Johnson, et al., 2015). This makes the need to use ICSI to achieve fertilization in the older group more evident. Moreover, poor sperm chromatin condensation resulting in increased DNA damage might have long-term effects not observed at the time of fertilization, biochemical pregnancy or ongoing pregnancy at 8 week ultrasound. These paternal effects may affect the development of live born children, as discussed below.

A study performed on DNA damage in sperm cells in mouse zygotes found that DNA repair is mediated by factors stored in the oocyte (Derijck, et al., 2008). DNA damage in spermatozoa is related to male ageing (Belloc, et al., 2014, Cocuzza, et al., 2008). In the literature, it is postulated that when oocyte quality is affected by age, repair mediating factors in the oocyte might be insufficient (Ben-Meir, et al., 2015, Derijck, et al., 2008). However, our results do not support this hypothesis in that spermatozoa of older men will lead to negative influence on embryo quality, BPR, OPR and miscarriage.

Although no paternal ageing effect on ongoing pregnancy rate at 8 weeks of gestational age was found in this study, other late genetic effects might be transferred to the offspring. The number of de-novo mutations in children of older fathers may be increased due to a combination of different mechanisms, such as DNA damage in spermatozoa, the increasing number of cell divisions during life combined with incorrect meiosis I, insufficient repair mechanisms because of lower testosterone concentrations in older men and environmental influences and life style (Bakshi, et al., 2001, Cocuzza, et al., 2008, Sakkas, et al., 1999).

De-novo mutations are known to largely originate during male gametogenesis, and their number increases with paternal age (Kong, et al., 2012, Veltman and Brunner, 2012). Most de-novo mutations occur outside of the gene and do not affect gene functioning. However, as more mutations occur at random in the genome this increases the chance that a mutation will affect a gene's functioning and thereby causes disorders such as intellectual disability. Genome sequencing recently indicated that de-novo mutations are the major cause of severe intellectual disability (Gilissen, et al., 2014). Others found associations between paternal age and DNA fragmentation in spermatozoa and fibroblast growth factor receptor 3 gene mutations associated with achondroplasia (Wyrobek, et al., 2006). In addition, epidemiological studies have clearly demonstrated an association between paternal age and the prevalence of autism spectrum disorders, schizophrenia and low educational attainment (D'Onofrio, et al., 2014, Humm and Sakkas, 2013). Others recently studied the relationship of paternal sperm DNA methylation and autism risk in offspring, in fathers of autistic children. They suggest based on their results that epigenetic differences in paternal spermatozoa may contribute to autism risk in offspring (Feinberg,

et al., 2015). Therefore, it may be that advanced paternal age does not have an impact on the probability of an ongoing pregnancy, but does have an impact on the risk of congenital abnormalities and neurodevelopment (Kovac, et al., 2013).

In conclusion, we found no significant influence of paternal age on OPR in assisted reproductive treatment at 8 weeks of gestational age. Further research should focus on paternal ageing influences on neonatal health and genetic disorders related to male infertility.

Supplementary tables

Supplemental table 1 Sub analysis of the IVF group - OR with 95% confidence interval (CI) of paternal age and of maternal age

		A-quality embryo	Biochemical pregnancy	Ongoing pregnancy
	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Maternal age (year)				
<35	2117	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-39	1315	0.79 (0.67-0.92)	0.70 (0.60-0.83)	0.65 (0.54-0.79)
≥40	320	0.76 (0.59-0.97)	0.54 (0.40-0.72)	0.48 (0.34-0.67)
Paternal age (year)				
<35	1548	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-44	1971	1.08 (0.93-1.25)	1.00 (0.85-1.17)	1.01 (0.85-1.21)
≥45	233	0.91 (0.68-1.23)	1.08 (0.78-1.49)	1.09 (0.76-1.55)

OR: odds ratio, IVF: in vitro fertilization, A-quality embryo: embryos with 0-20% fragmentation and containing 7, 8 or 9 blastomeres on the 3th day after ovum pick up.

Supplemental table 2 Sub analysis of the ICSI group - Adjusted OR with 95% confidence interval (CI) of paternal age and of maternal age

		A-quality embryo	Biochemical pregnancy	Ongoing pregnancy
	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Maternal age (year)				
<35	2409	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-39	766	0.78 (0.65-0.93)	0.72 (0.60-0.87)	0.77 (0.62-0.94)
≥40	124	0.60 (0.41-0.88)	0.35 (0.22-0.55)	0.30 (0.17-0.54)
Paternal age (year)				
<35	1528	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-44	1368	0.94 (0.80-1.11)	0.98 (0.83-1.15)	0.95 (0.80-1.13)
≥45	403	0.97 (0.76-1.24)	1.04 (0.81-1.35)	1.01 (0.76-1.33)

OR: odds ratio, ICSI: intracytoplasmic sperm injection, A-quality embryo: embryos with 0-20% fragmentation and containing 7, 8 or 9 blastomeres on the 3th day after ovum pick up.
Note that the adjusted OR's are adjusted for ICSI with ejaculated and non-ejaculated sperm and the other variable in model by using multivariable logistic regression analysis.

Supplemental table 3 Sub analysis of the ICSI group with non-ejaculated sperm - OR with 95% confidence interval (CI) of paternal age and of maternal age

		A-quality embryo	Biochemical pregnancy	Ongoing pregnancy
	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Maternal age (year)				
<35	668	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-39	264	0.61 (0.44-0.83)	0.78 (0.56-1.08)	0.88 (0.61-1.25)
≥40	46	0.49 (0.26-0.91)	0.42 (0.20-0.90)	0.34 (0.13-0.88)
Paternal age (year)				
<35	326	1.00 (reference)	1.00 (reference)	1.00 (reference)
35-44	412	1.23 (0.90-1.67)	0.85 (0.62-1.17)	0.84 (0.60-1.19)
≥45	240	1.27 (0.88-1.83)	0.85 (0.58-1.25)	0.82 (0.54-1.23)

OR: odds ratio, ICSI: intra cytoplasmic sperm injection, A-quality embryo: embryos with 0-20% fragmentation and containing 7, 8 or 9 blastomeres on the 3th day after ovum pick up

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7

General discussion

In this thesis various aspects of safety and efficacy of assisted reproductive techniques (ART), especially regarding non-obstructive azoospermia (NOA) and testicular sperm extraction with intracytoplasmic sperm injection (TESE-ICSI) treatment are explored. This thesis addresses questions from both the couples who are considering treatment, as well as the physicians treating male infertility. In this chapter the main findings are summarized and discussed, including their implications for daily practice. Finally, suggestions on which future research should focus are pointed out.

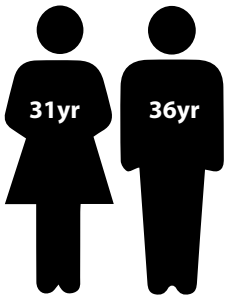
Basically, this thesis is written, to help patients and their physicians in their journey in ART. So, let's start from that point of view. Imagine, **Couple X** and **Doctor Y**.

TESE & TESE-ICSI

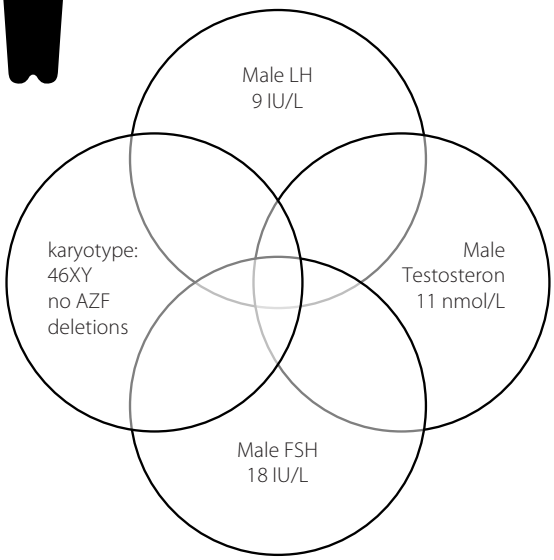
When Couple X is referred to the fertility clinic, they meet Doctor Y (Box 1). After complete andrologic evaluation including medical history, physical examination and blood tests, Doctor Y tells the couple that Mr. X will need a TESE procedure in order to obtain spermatozoa for ICSI. From the literature it's known that the chance of successful sperm retrieval is about 50% (Chan and Schlegel, 2000, Colpi, et al., 2005, Tournaye, 2010). A number of prediction models for successful sperm retrieval in TESE have been developed but none of them has been externally validated (Boitrelle, et al., 2011, Ramasamy, et al., 2013, Samli and Dogan, 2004, Tsujimura, et al., 2004).
In order to counsel the couple appropriately, we developed and externally validated a prediction model for obtaining spermatozoa with TESE in men with NOA, as described in **Chapter 2**. Our model demonstrated that a higher male age, higher values for testosterone and lower values for follicular stimulating hormone (FSH) and luteinizing hormone (LH) are predictive for successful sperm retrieval. Diagnosis of idiopathic NOA and an AZF-c deletion were predictive for unsuccessful sperm retrieval.

As mentioned, male age is one of the parameters included in our prediction model. One should realize that including male age into the model did improve the fitness of our model. Although there is a positive correlation between higher male age and successful sperm retrieval, we later on discuss potential negative effect of male age on the child health. However, one should take into account that in our development data set male age had a range between 22 and 72 years but only 4% (37/918) of them were above 45 years old. Moreover it is possible that there is a selection bias based on severity of testicular dysfunction. In other words, it is imaginable that in younger men with severe testicular dysfunction it is still more likely to find just enough ejaculated spermatozoa to perform ICSI and will not yet present azoospermia. While when getting older more often these males have developed azoospermia. With other words, when a young man presents with

Box 1 Couple X



- Together for 6 years
- Married for 2 years
- Trying to conceive for 1.5 year
- Mr. X is recently diagnosed with (idiopathic) NOA



azoospermia he might probably suffer from a more severe form of a spermatogenesis disorder. The fact that older men still can produce sperm does not reflect the superior quality of them.

The predictive parameters found in our model confirmed that hormones are involved in the disturbed spermatogenesis.

After knowing the chance of obtaining spermatozoa (Box 2) it is also important to know the chance to conceive. Therefore, as described in **Chapter 3,** we developed and externally validated a prediction model for live birth after TESE-ICSI. Our model demonstrated that a lower female age, a first (versus subsequent) TESE-ICSI cycle, lower male LH, higher male testosterone, availability of motile spermatozoa for ICSI and having obstructive azoospermia as an initial diagnosis were predictive for a live birth.

Box 2 Couple X

Probability of obtaining spermatozoa in TESE = 1/ (1+ exp (–(β))) where
β= -1.009 + (male age * 0.058) + (LH x -0.115) + (LH2 * 0.001) + (FSH * -0.019) + (testosterone * 0.034) + (AZF-c deletion * -1.480) + (idiopathic NOA * -0.855).

Mister X
Male age: 36
LH: 9 IU/L
FSH: 18 IU/L
Testosterone: 11 nmol/L
AZF-c deletion: no (0)
Ideopathic NOA: yes (1)

Following our predictive model above the chance to find sperm for Mr. X is 33%

At this point, Doctor Y is able to involve Couple X in the process of making decisions about their personal chances for conception (Box 3) so that Couple X can decide for their own whether they want to join the 'ART' journey. This is called "shared decision making" (Legare, et al., 2010). Using shared decision Doctor Y and Couple X can assess the burden of the TESE and ICSI treatment and on the other hand the chance of obtaining spermatozoa and finally achieving a live birth. Several studies demonstrate that patients who are involved in decision making are less prone to experience decisional conflict and regret (Bastings, et al., 2014, Legare, et al., 2010). Using our model Doctor Y is able to inform Couple X about their actual chances.

Unfortunately, our developed prediction models have also some limitations. Both models have a retrospective study design and lack complete data on potentially contributing factors such as male BMI. The negative influence of male obesity on reproductive outcome has been reported in terms of impaired blastocyst development and reduced clinical pregnancy rate and live birth rate as well as an increased risk of miscarriage (Bakos, et al., 2011). Others describe that sperm retrieval rates were similar in normal weight and obese man, but a lower male BMI predicted a higher chance of clinical pregnancy after micro dissection TESE-ICSI (Ramasamy, et al., 2013). Obesity may alter the microenvironment inducing epigenetic changes (Milagro, et al., 2013), which in turn impairs embryo development in couples with male partners suffering from azoospermia.

It is assumed that obesity deregulates the hypothalamic pituitary gonadal axis, resulting in aberrant reproductive hormones such as reduced testosterone and elevated oestrogen levels which are in turn associated with impaired spermatogenesis (Fejes, et al., 2006,

Box 3 Couple X

Probability of live birth after TESE-ICSI = exp(-β)/(1+exp(-β)) where β=7.659 + (female age * 0,442) + (female age^2 * -0,008) + (cycle number * -0,278) + (motility*0,608) + (testosterone*0,026) + (LH*-0,06) + (OA/NOA*0,269))

Couple X
Female age: 31
Cycle number: 1
Testosterone: 11 nmol/L
LH: 9 IU/L
OA/NOA: NOA (0)

In case sperm is found in TESE, the chance of a live birth for Couple X, following our prediction model above, is: 17% in their first TESE-ICSI cycle if there are motile spermatozoa available for injection of the oocyte and 10% if viable but immotile spermatozoa are used.

Strain, et al., 1982). In males with NOA the hypothalamic pituitary axis is generally already deregulated but obesity might also contribute to low testosterone levels. An inverse relationship between male BMI and motility of sperm as well as sperm chromatin integrity has been found in normal healthy men (Kort, et al., 2006). Male obesity may not only influence hormones and reproductive outcome but also affect the offspring according to a study which found that paternal obesity is associated with insulin-like Growth Factor 2 hypomethylation in newborns (Soubry, et al., 2013).
Both our models have limitations in their predictive value, with an area under the curve (AUC) of 0.69 in the prediction model for obtaining spermatozoa and an AUC of 0.62 for the prediction model for live birth. Moreover, the predicting model for live birth includes the parameter motility of the spermatozoa used for ICSI, information that is not available before the start of the TESE procedure. In the counselling of couples one should discuss both options i.e. with or without motile spermatozoa used for ICSI. For the future it would be valuable to develop and validate one integrated prediction model for predicting the chance of a live birth before undergoing a TESE procedure.

Health of offspring

Using immature spermatozoa for fertilization of oocytes in TESE-ICSI might have consequences for the children born after TESE-ICSI. Mr. and Mrs. X heard about the moratorium which was set on ICSI with surgical retrieved sperm due to concerns about the possible short and long term effects on the health of children. Couple X would like to be informed about these possible effects on child's health.
In **Chapter 4** these issues were addressed. We found no significant difference in the prevalence of major birth defects between the TESE group (5.9%) and the PESA group (6.9%). We also studied whether or not several maternal and treatment related factors – maternal age, maternal smoking behaviour, fresh or frozen-thawed sperm, fresh or frozen-thawed embryos and embryo quality –had a significant effect on the prevalence of birth defects. This was not the case. In addition to the results described in chapter 4, we studied the birth defects in relation to paternal age. The paternal age of children with or without a major birth defect did not differ significantly in the PESA group, in the TESE group the paternal age of children with a major birth defect was significantly lower. This might reflect a more severe spermatogenesis disorder in younger NOA men compared with older NOA men as discussed previously. Moreover, we performed an observational prospective cohort study to behavioural, cognitive and motor performance and growth parameters of five-year-old children born after TESE-ICSI as described in **Chapter 5**. Four children (3.8%) of the 'five-years-old cohort' presented with developmental problems. Two of them were already diagnosed with a form of autism (Pervasive Developmental Disorder-Not Otherwise Specified). Two children had developmental problems based on two or more of our behavioural, cognitive and motor assessments. In addition to the results described in chapter 5, we found no significant difference in paternal age of the children with or without a developmental problem.
Our 'five-years-old cohort' assessed significantly better on behavioural and cognitive performance than general population. The 'five-years-old cohort' assessed significantly worse on motor performance compared with general Dutch population. Although there is a significant difference compared with general Dutch population, the mean total test score of our cohort is still within the normal range and is therefore less clinically relevant. Our results concerning the safety of TESE-ICSI regarding long term effects on development and health in children born after these procedures, seems therefore reassuring. Doctor Y is now able to tell Couple X about the prevalence of birth defects and the development of the children born after TESE-ICSI.

In the meantime the Minister of Health, Welfare and Sport lifted the moratorium on TESE-ICSI in June 2014 after the presentation of a preliminary report about the safety of treatment (NVOG, 2013, Staatscourant, 2014). After this decision, beside the fertility centres of Radboud university medical center and Academic Medical Center other fertility centres

can to offer TESE-ICSI treatment as long as they adhere to the professional Quality Standard. To collaborate in TESE care, Radboud university medical center and Academic Center initiated biannual TESE-NL meetings for all Dutch fertility centres which are (planning to) provide TESE (-ICSI). During these meetings all fertility centres agreed to register all TESE procedures and whether or not there were complications during or after treatment.

Paternal ageing

In this given example of Couple X, Mrs. X is 31 and Mr. X is 36 years old. What if Mr. X would have been a lot older, e.g. 68 years? Doctor Y knows that women older than 35 years have a higher risk of spontaneous abortion, pregnancy complications and chromosomal abnormalities (de la Rochebrochard and Thonneau, 2002), and also the chance of getting pregnant decreases with women's age. In the Netherlands the participation in reproductive programs for women is available till 45 years, however only reimbursed for women aged up to 42. But what about ageing men, what are their chances in ART?

In **Chapter 6**, we studied the paternal age effect on reproductive outcome after different ART treatments in terms of embryo quality, biochemical pregnancy and ongoing pregnancy. We did not find a paternal age effect for these criteria. Subgroup analyses based on IVF, ICSI and ICSI with non-ejaculated spermatozoa, nearly identical results were found. Although the results of this study do not support our hypothesis of a negative effect on ART outcome due to paternal ageing, this subject should be studied more extensively. As stated in the introduction of this thesis, male age for parenthood has increased in the last decade. In the Netherlands in 2000, 11.1% (n=22981) of the children born had a father over 40 years old; in 2012 this percentage was 16.4% (n=28 888) (CBS, 2011). No paternal ageing effect on ongoing pregnancy was found in this study. This doesn't mean that paternal age has no effect. It would be of great interest to link paternal age at conception to data on live births including data on the presence or absence of congenital abnormalities or developmental delay. As stated in the introduction, epidemiological literature demonstrates an association between paternal age and the prevalence of autism spectrum disorders, schizophrenia and low educational attainment in offspring (D'Onofrio, et al., 2014, Humm and Sakkas, 2013).

We suspect that several biological processes are influenced by paternal ageing. *De novo* mutations are known to largely originate during male gametogenesis and their number increases with paternal age (Crow, 2000, Kong, et al., 2012). Recent studies using exome and whole genome sequencing described that many *de novo* mutations in children with intellectual disability had a paternal origin (Crow, 2000, Glaser, et al., 2000, Hehir-Kwa, et al., 2011, Moloney, et al., 1996, Vissers, et al., 2010). About 80% of the copy number variations originated from the fathers (Hehir-Kwa, et al., 2011). Results of next generation DNA

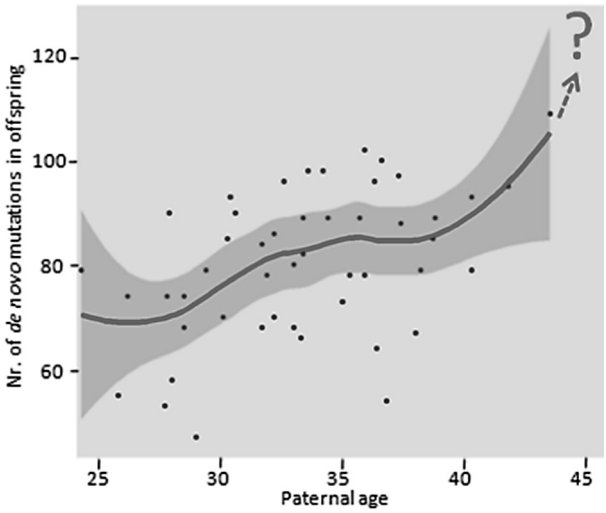


Figure 1 Relation between paternal age, number of de novo mutations and genetic disease in offspring, based on pilot data (Gilissen, et al., 2014)

sequencing techniques confirm that humans have an exceptionally high mutation induction rate per generation (Vissers, et al., 2010). Most *de novo* mutations do not lead to a congenital abnormality, as most mutations occur outside of gene and do not affect gene functioning. However, as more mutations occur random in the genome this increases the chance of mutation affecting gene functioning and thereby causing disorders such as intellectual disability.

Although the association between the number of de novo mutations and increased paternal age has been reported, so far no data are available on the number of *de novo* mutations in offspring from fathers over 45 (Hehir-Kwa, et al., 2011, Kong, et al., 2012). It is therefore currently unknown whether paternal age effects remains linear or become exponential after a certain age (Figure 1), as is the case for *de novo* chromosomal abnormalities in women over 35 (Schmidt, et al., 2012). Besides, it is still unknown whether the use of IVF or ICSI (with ejaculated or non-ejaculated sperm) influences the number of de novo mutations in offspring. Further research should focus on these two aspects.

Conclusion

In this thesis various aspects of safety and efficacy in severe male infertility were studied. Two prediction models for couples undergoing TESE-ICSI were developed and validated. Moreover, the safety of ICSI with testicular spermatozoa was studied and seems to be a safe treatment regarding the offspring up to the age of five. No increased numbers of birth defects or developmental problems were found. Finally, no paternal ageing effect on reproductive outcome in terms of embryo quality, biochemical pregnancy and ongoing pregnancy after ART could be demonstrated.

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CBS (2011) One out of six fathers is over 40 at birth of their child.

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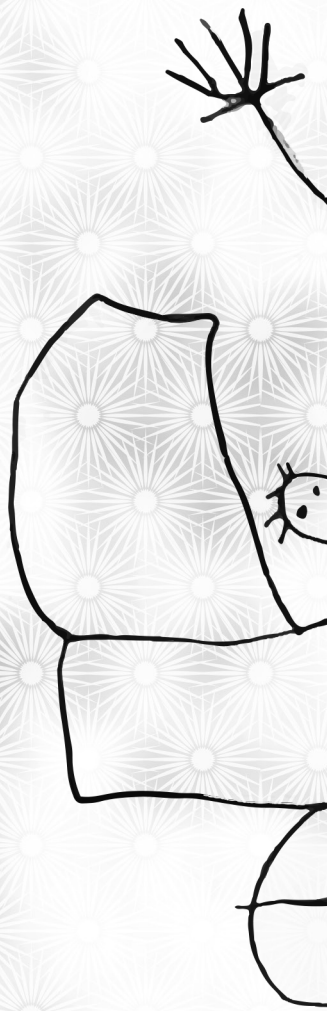
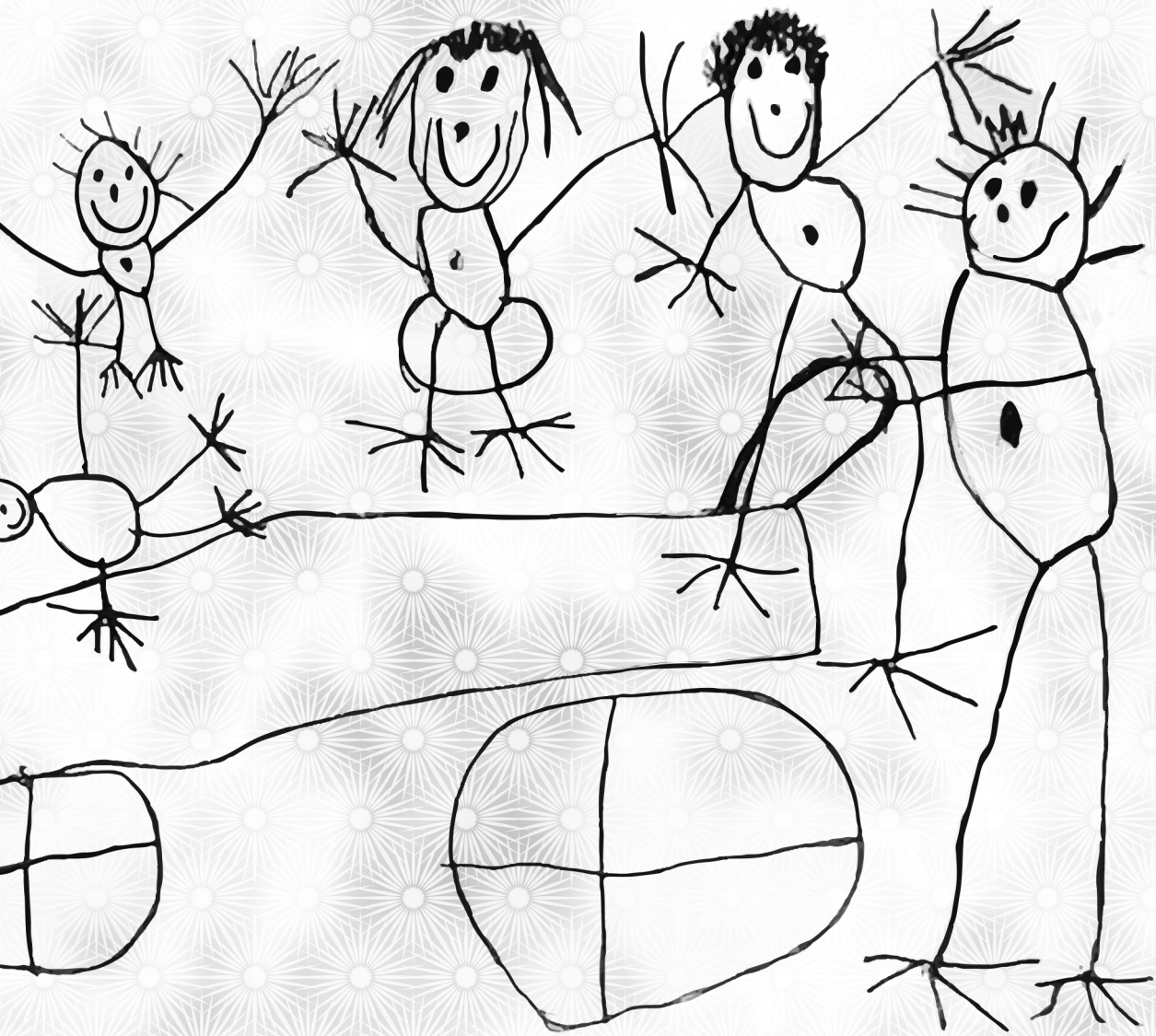


8

Summary | Samenvatting



Summary

In this thesis, we describe the research we have performed in order to improve the counselling of couples with severe male infertility. We focus primarily on the safety and efficacy of fertility treatments for couples facing azoospermia and need testicular sperm extraction with intracytoplasmic sperm injection (TESE-ICSI) treatment. The introduction (**Chapter 1**) addresses the medical background of azoospermia, the introduction of assisted reproduction techniques in a historical perspective and the available knowledge on the safety of the TESE-ICSI. In addition, the potential risks of an increased paternal age are discussed. Finally, we formulated a number of research questions.

In **Chapter 2**, we examine what factors are predictive for obtaining spermatozoa with TESE in men with non-obstructive azoospermia. We studied the results of 918 men with a non-obstructive azoospermia (NOA) who underwent a first TESE procedure. After analysis we found that the probability of obtaining spermatozoa with TESE was associated with a higher male age, a higher testosterone and a lower follicular stimulating hormone and luteinizing hormone. In addition, an idiopathic NOA or having an AZF-c deletion on the Y chromosome had a negative effect on the probability of obtaining spermatozoa with TESE. The developed prediction model had an area under the curve (AUC) of 0.69 and was tested on an external patient population in which 453 TESE procedures were performed. The model was externally validated and had an AUC of 0.65. If sperm retrieval was successful, the couple can continue treatment with TESE-ICSI in order to try to conceive. Therefore, we investigated in **Chapter 3** what factors are predictive of the likelihood of a live birth after TESE-ICSI treatment. We studied 526 couples with fertility problems based azoospermia who underwent a total of 1006 TESE-ICSI treatment cycles. After analysis we found that the probability of a live birth was associated with a lower female age, a first treatment cycle, a low male luteinizing hormone, a high level of male testosterone, the availability of motile spermatozoa for injection of the oocytes, and having an obstructive azoospermia as an initial diagnosis. The prediction model we developed this had an AUC of 0.62 and was tested on an external patient population with 289 couples who underwent 553 cycles. The model was externally validated and had an AUC of 0.67.

Chapter 4 describes the results of our study regarding the prevalence of birth defects in children born after ICSI treatment with percutaneous epididymal sperm aspiration (PESA) and TESE-ICSI. We studied the birth defects in 406 children who were born after PESA-ICSI in 237 children who were born after TESE-ICSI. The prevalence of 'major' birth defects was 6.9% in the PESA group and 5.9% in the TESE group, this was not significantly different. In addition, we investigated whether maternal or treatment factors (including maternal age, maternal smoking, using fresh or frozen spermatozoa, transferring fresh or frozen embryos and embryo quality) influenced the risk of a birth defect. This could not be demonstrated.

Chapter 5 describes the study in which we investigated whether children born after TESE-ICSI have behavioural, cognitive, motor and / or physical problems at age five. We

evaluated the development in 404 children born after TESE-ICSI at birth, one year and four years of age, and evaluated the development of 103 five-year-olds with behavioural, cognitive and motor performance tests and growth parameters. Four children (3.8%) of the 'five-years-old cohort' had developmental problems. Two of them were previously diagnosed with a form of autism (Pervasive Developmental Disorder-Not Otherwise Specified). Two children had developmental problems based on our behavioural, cognitive and motor assessments. Our 'five-years-old cohort' assessed significantly better on behavioural and cognitive performance and significantly worse on motor performance than general population. Our results concerning the safety of TESE-ICSI regarding long term effects on development and health in children born after these procedures, seems therefore reassuring.

Chapter 6 describes the results of a study on the influence of paternal age on the availability of top quality embryo for transfer, biochemical pregnancy rate, ongoing pregnancy rate and miscarriage after the application of assisted reproduction techniques. We studied the results of 7051 first in vitro fertilization (IVF) and ICSI treatments. After analysis, we found no significant effect of paternal age on the availability of top quality embryos for transfer, biochemical pregnancy rate, ongoing pregnancy rate and / or miscarriage after use of assisted reproduction techniques. In subgroup analyses based on IVF, ICSI and ICSI with non-ejaculated sperm, nearly identical results were found.

In **Chapter 7** of this thesis, we discuss the main conclusions of our studies. With the use of an example case, we described the applicability of the prediction models developed by us and the implications for the practice for both the doctor and the patient. ICSI with testicular sperm obtained by TESE seem to be a safe treatment according to study regarding the development of five-year-olds born after TESE-ICSI. We could not demonstrate a paternal age effect on the outcome of assisted reproduction techniques.

Samenvatting

In dit proefschrift beschrijven we het onderzoek dat we hebben verricht om paren met ernstige mannelijke subfertiliteit beter te kunnen counselen. Onze focus ligt voornamelijk op de veiligheid en de effectiviteit van vruchtbaarheidsbehandelingen bij paren met azoöspermie waarbij een testiculaire sperma extractie met intracytoplasmatische sperma injectie (TESE-ICSI) behandeling wordt verricht. In de introductie (**Hoofdstuk 1**), wordt de medische achtergrond van azoöspermie, de introductie van geassisteerde voortplantingstechnieken in een historisch perspectief en de beschikbare kennis over de veiligheid van de TESE-ICSI behandeld. Daarnaast worden de potentiële risico's van een toegenomen leeftijd van de man besproken. Aan de hand hiervan formuleerden we een aantal onderzoeksvragen.

In **Hoofdstuk 2** onderzoeken we welke factoren voorspellend zijn voor de kans op het vinden van zaad bij TESE bij mannen met een niet obstructieve azoöspermie (NOA). We bestudeerden de resultaten van 918 mannen met een NOA die een eerste TESE ingreep ondergingen. Na analyse bleek dat de kans op het verkrijgen van zaad bij TESE was geassocieerd met een hogere leeftijd, een hoger testosteron, een lager folliculair stimulerend hormoon en luteïniserend hormoon. Daarnaast hadden een ideopathische NOA en het hebben van een AZF-c deletie op het Y chromosoom een negatief effect op de kans op het verkrijgen van zaad bij TESE. Het predictie model wat we hiermee ontwikkelden had een 'area under the curve' (AUC) van 0.69 en werd getest op een externe patiënten populatie waarin 453 TESE's werden verricht. Het model werd hierdoor extern gevalideerd en had een AUC van 0.65. Als er bij deze paren zaad werd gevonden, dan konden zij behandeld worden met TESE-ICSI in de hoop dat hun kinderwens in vervulling kon gaan. Daarom hebben we in **Hoofdstuk 3** onderzocht welke factoren vervolgens voorspellend zijn voor de kans op een levend geboren kind na TESE-ICSI behandeling. We bestudeerden 526 paren met een vruchtbaarheidsprobleem op basis van azoöspermie die in totaal 1006 TESE-ICSI behandelcycli ondergingen. Na analyse bleek dat de kans op een levend geborene was geassocieerd met een lagere leeftijd van de vrouw, een eerste behandel cyclus, een laag mannelijk luteïniserend hormoon, een hoog mannelijk testosteron, de beschikbaarheid van bewegende zaadcellen voor ICSI en de verdenking op een obstructieve azoöspermie bij het eerste onderzoek. Het predictie model wat we hiermee ontwikkelden had een AUC van 0.62 en werd getest op een externe patiënten populatie met 289 paren die 553 behandelcycli ondergingen. Het model werd hierdoor extern gevalideerd en had een AUC van 0.67.

Hoofdstuk 4 beschrijft de resultaten van onze studie naar de prevalentie van aangeboren afwijkingen bij kinderen die geboren zijn na behandeling met percutane epipidymale sperma aspiratie (PESA)-ICSI en TESE-ICSI. We bestudeerden de aangeboren afwijkingen in 406 kinderen die geboren waren naar PESA-ICSI en in 237 kinderen die geboren waren na TESE-ICSI. De prevalentie van 'major' aangeboren afwijkingen was 6.9% in de PESA

groep en 5.9% in de TESE groep, dit was niet significant verschillend. Daarnaast onderzochten we of maternale of behandelingsfactoren (o.a. leeftijd van de moeder; rookgedrag van de moeder; het gebruik van vers of ingevroren zaad; het terugplaatsen van verse of ingevroren embryo's; de embryokwaliteit) invloed hadden op de kans op een aangeboren afwijking. Dit werd niet aangetoond.

Hoofdstuk 5 beschrijft de studie waarin we hebben onderzocht of kinderen die geboren zijn na behandeling met TESE-ICSI gedrags, cognitieve, motorische en/of lichamelijke problemen hebben op vijfjarige leeftijd. We bestudeerden de ontwikkeling van 404 kinderen geboren na TESE-ICSI bij de geboorte, op 1-jarige en 4-jarige leeftijd. Daarnaast onderzochten we het gedrag, de intelligentie, de motorische en de lichamelijke ontwikkeling van 103 vijfjarige kinderen uit deze groep. Vier kinderen (4%) van de onderzochte vijfjarigen hadden problemen in de ontwikkeling. Twee van hen waren al gediagnosticeerd met een vorm van autisme (PDD-NOS). De twee andere kinderen hadden problemen in de ontwikkeling gebaseerd op onze testen. Onze groep onderzochte 5-jarige scoorden significant beter op gedrag en intelligentie en significant slechter op motoriek in vergelijking met een algemene groep kinderen. Onze resultaten met betrekking tot de ontwikkeling van kinderen geboren na TESE-ICSI en de veiligheid van de behandeling zijn daarom geruststellend.

In **Hoofdstuk 6** worden de resultaten beschreven van een studie naar de invloed van de leeftijd van de man op de beschikbaarheid van een topkwaliteit embryo voor terugplaatsing, het optreden van een biochemische zwangerschap, doorgaande zwangerschap en/of miskraam na de toepassing van geassisteerde voortplantingstechnieken. We bestudeerden de resultaten van 7051 eerste in vitro fertilisatie (IVF) / ICSI behandelingen. Na analyse vonden we geen significant effect van paternale leeftijd op de beschikbaarheid van een topkwaliteit embryo voor terugplaatsing, het optreden van een biochemische zwangerschap, doorgaande zwangerschap en/of miskraam na toepassing van geassisteerde voortplantingstechnieken. Ook in subgroep analyses waarbij we hebben gekeken naar IVF, ICSI of ICSI met chirurgisch verkregen zaad vonden we vrijwel dezelfde resultaten, met andere woorden geen invloed van paternale leeftijd op de behandeluitkomsten.

In **Hoofdstuk 7** van dit proefschrift gaan we in op de belangrijkste conclusies die we hebben kunnen trekken uit dit onderzoek. We hebben de toepasbaarheid van de door ons ontwikkelde predictiemodellen beschreven en de implicaties voor de praktijk voor zowel de dokter als de patiënt aan de hand van een voorbeeld. ICSI behandeling met testiculair verkregen zaad door middel van TESE lijkt een veilige behandeling als we naar de resultaten van onze studie naar de ontwikkeling van vijfjarige kinderen kijken. We hebben geen paternaal leeftijdseffect kunnen aantonen op de uitkomsten van geassisteerde voortplantingstechnieken.

Appendix

Abbreviations

Portfolio

List of publications

Dankwoord

Curriculum Vitae

Abbreviations

ART	assisted reproductive techniques
AZF	azoospermia factor
BPR	biochemical pregnancy rate
CBCL	child behaviour checklist
CI	confidence interval
DET	double embryo transfer
DNA	desoxyribo nucleic acid
ET	embryo transfer
FSH	follicular stimulating hormone
HCG	human chorionic gonadotrophin
ICSI	intracytoplasmic sperm injection
IQ	intelligence quotient
IVF	in vitro fertilization
LH	luteinizing hormone
M-ABC	movement assessment battery for children
MESA	microsurgical epididymal sperm aspiration
OHSS	ovarian hyperstimulating syndrome
OPR	ongoing pregnancy rate
OPU	ovum pick up
OR	odds ratio
PESA	percutaneous epididymal sperm aspiration
SET	single embryo transfer
TESE	testicular sperm extraction
TRF	teacher report form
WPPSI	wechsler preschool and primary scale of intelligence

Institute for Health Sciences
Radboudumc

PHD Portfolio

Name PhD candidate: A.M. Meijerink
Department: Obstetrics & Gynaecology
Graduate School: Radboud Institute for Health Sciences

PhD period: 15-08-2013 – 03-06-2016
Promotor(s): Prof. dr. D.D.M. Braat, Prof. dr. ir. J.A. Veltman
Co-promotor(s): Dr. K. Fleischer, Dr. L. Ramos

		Year(s)	ECTS
	TRAINING ACTIVITIES		
a)	Courses & Workshops		
-	BROK course	2014	1
-	RIHS introduction course	2014	1
-	Erasmus Summer Programme 'Markers and prediction research'	2014	1
-	Statistics refreshing course for PhD students	2014	1
-	Academic writing course	2015	3
-	ART of presenting science course	2015-2016	1.5
b)	Seminars & lectures		
-	Visiting lecture 'Psychology of fertility problems'	2014	0.1
-	TESE-NL meeting, oral presentation	2015	0.25
-	IPN ESHRE review, oral presentation	2015	0.25
c)	Symposia & congresses		
-	Symposium of fertility studies, Antwerp	2015	0.25
-	Symposium Dutch board of fertility physicians	2015	0.25
-	ESHRE Lisbon, oral presentation	2015	0.5
-	Nation fertility division day	2016	0.25
-	ESHRE Helsinki, 2 oral presentations	2016	1.0
-	ESHRE Helsinki, poster presentation	2016	0.5
d)	Other		
-	Journal club	2013-2016	1
	TEACHING ACTIVITIES		
e)	Lecturing		
-	OBGYN residents teaching	2015	0.25
f)	Supervision of internships / other		
-	Supervision research student	2014	1
-	Supervision research student	2014	1
TOTAL			**15.1**

Radboud University Radboudumc university medical center

List of publications

Meijerink AM, Cissen M, D'Hauwers KW, Meissner A, van der Weide N, Mochtar MH, de Melker AA, Ramos L, Repping S, Braat DD, Fleischer K, van Wely, Prediction model for obtaining spermatozoa with testicular sperm extraction in men with non-obstructive azoospermia; Hum Reprod. 2016 Sep;31(9):1934-41.

Meijerink AM, Cissen M, Mochtar MH, Fleischer K, Thoonen I, de Melker AA, Meissner A, Repping S, Braat DD, van Wely M, Ramos L, Prediction model for live birth in ICSI using testicular extracted sperm. Hum Reprod. 2016 Jul Sep;31(9):1942-51.

Meijerink AM, Ramos L, Fleischer K, Veltman JA, Hendriks JC, Braat DDM, Influence of paternal age on ongoing pregnancy rate at eight weeks' gestation in assisted reproduction. Reprod Biomed Online. 2016 Jan;32(1):96-103.

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Meijerink AM, Rijssel van RH, Linden van der PJQ, Tissue composition of the vaginal wall in women with pelvic organ prolapsed, Gynecol Obstet Invest. 2013;75(1):21-7.

Dankwoord

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Leden van de manuscript commissie, geachte prof. C. Noordam, prof. dr. M.J.C. Eijkemans en prof. dr. P. de Sutter, hartelijk dank voor het beoordelen van het manuscript. Alle leden van de corona wil ik graag bedanken voor het vervullen van hun taak als opponent bij de verdediging.

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Wat moet een promovendus zonder een paar goede studenten? Reinoud en Ilse wat had ik het met jullie getroffen, bedankt voor jullie inzet en fanatisme.

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Curriculum Vitae

Aukje Meijerink werd op 25 april 1987, als oudste van drie kinderen, geboren te Deventer en groeide op in het Overijsselse Lettele. De middelbare school doorliep zij aan het Geert Groote College (later: Etty Hillesum Lyceum) te Deventer waaraan zij in 2005 haar VWO diploma behaalde. Na te zijn ingeloot voor de studie geneeskunde verhuisde zij naar het hoge noorden om aan de Rijks universiteit Groningen te gaan studeren. Tijdens haar studie maakte Aukje twee buitenlandse uitstapjes, eenmaal naar Bangkok (Thailand) voor een IFMSA stage kindergeneeskunde en eenmaal naar Kondoa (Tanzania) voor een coschap sociale/tropen geneeskunde. Echter Aukje raakte in het bijzonder geïnteresseerd in de gynaecologie. Na twee reguliere coschappen gynaecologie in het UMCG (Groningen) en in de Isala Klinieken (Zwolle) besloot zij ook een semi arts stage en een wetenschappelijke stage binnen de gynaecologie te volgen in het Deventer Ziekenhuis onder leiding van dr. P.J.Q. van der Linden. Tijdens deze wetenschappelijke stage kwam zij voor het eerst in aanraking met pathologie, immuunhistochemie en het doen van onderzoek, dit resulteerde in een eerste publicatie. In 2012 nam zij haar artsenbul in ontvangst, waarna zij als ANIOS gynaecologie in het Jeroen Bosch Ziekenhuis (Den Bosch) aan de slag ging. Na een leuke, leerzame en tijd in Den Bosch was zij helemaal overtuigd, ze wilde gynaecoloog worden. Aukje startte in 2013 als fertiliteitsarts en arts-onderzoeker in het Radboudumc (Nijmegen). Aanvankelijk betrof het onderzoek de follow up van de ontwikkeling van kinderen geboren na ICSI-TESE echter als snel groeide dit uit tot een promotietraject onder leiding van prof. dr. D.D. M. Braat, prof. dr. ir. J.A. Veltman, dr. K. Fleischer en dr. L. Ramos. Het proefschrift dat nu voor u ligt is hiervan het resultaat. Per 1 februari 2017 zal Aukje starten met de opleiding tot gynaecoloog in cluster Nijmegen, in ziekenhuis Gelderse Vallei te Ede.